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The Role of Astrocyte and Purinergic Signaling in RTN Chemoreception

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Body of Abstract

Central chemoreception is the mechanism by which the brain regulates breathing in response to changes in tissue pH/CO_2. It is particularly important during sleep and its disruption has been associated with certain pathologies, including central sleep apnea and central congenital hypoventilation syndrome. A region of the brainstem called the retrotrapezoid nucleus (RTN) is thought to be an important site of central chemoreception.

Recent evidence suggests that RTN chemoreception involves two mechanisms. The first appears to be H^+-mediated activation of neurons via inhibition of pH-sensitive potassium channels. More recently, a second sensing mechanism has been identified; paracrine release of ATP in the RTN region that acts on P2 receptors. It has been postulated that astrocytes in the region release ATP, as they have been found to release ATP in other brain regions in response to various stimuli. However, the mechanisms by which astrocytes sense CO2 and/or pH are not known.

Astrocytes in many brain regions are known to express Kir4.1 and/or Kir4.1-Kir5.1 potassium channels, which are highly pH sensitive. In addition, Kir4.1-Kir5.1 is expressed in the RTN. I hypothesize that RTN astrocyte sense CO_2/H^+ by inhibition of Kir4.1-Kir5.1 channels, which results in membrane depolarization. I also hypothesize that ATP released via connexin hemichannels in response to CO_2/H^+ acts on P2-receptors on RTN neurons to increase neuronal action potential firing, and that this purinergic mechanism of chemoreception is restricted to the ventral surface, affecting primarily RTN neurons. The evidence described in this thesis supports four main conclusions; (1) Kir4.1/Kir5.1 channels, and the

sodium bicarbonate cotratransporter, are the mechanism of RTN astrocyte chemosensitivity, (2) CO_2-driven purinergic signaling in the RTN is required for complete chemosensation *in vivo* and *in vitro*, (3) CO_2-evoked ATP release is dependent on connexin hemichannels and (4) these purinergic mechanisms are specific to the RTN.

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Dedication

I would like to dedicate this thesis to my beautiful wife Jelena.

Table of Contents

List of Figures

Chapter 1 Introduction

Breathing is a physiological phenomenon that begins at birth and continues, relatively uninterrupted, until the end of life. For terrestrial animals, like humans, breathing occurs by moving air in and out of the lungs. The much needed oxygen (O_2) diffuses into the blood via the pulmonary epithelial cells, and the waste product, carbon dioxide (CO_2), likewise diffuses out.

The drive to breathe must adjust to metabolic demand, with increases occurring during activities like exercise or holding one's breath. This drive to breathe is even present during periods of relaxation or sleep, as is evident by our continuous respiratory activity. Conversely, disruption of the drive to breathe is thought to be a cause of death for people with certain pathologies, including epilepsy and stroke (Davis, Billings et al. 2013, Richerson, Buchanan 2011). In other cases compromised respiratory drive does not lead to death, but severely impacts quality of life. Such is the case in central congenital hypoventilation syndrome and the more common sleep apnea (Shea 1997, Dempsey, Smith et al. 2012).

Central chemoreception is the homeostatic process by which the central nervous system senses CO_2 in order to adequately maintain O_2/CO_2 levels and is a major component of respiratory drive. Although the study of central chemoreception dates back at least half a century, it's cellular and molecular mechanism remains incompletely understood. Better understanding of these mechanisms could lead to new therapies to improve the quality of life for people afflicted with the aforementioned diseases.

In addition, I would like to point out another important reason for the study of central chemoreception, breathing and autonomic behaviors in general. We are in the advent of modern neuroscience. Today the neuroscience community is, more than ever, discussing the best ways to understand how the brain functions at every level. The new funding mechanisms the B.R.A.I.N. initiative in the United States and the Human Brain Project in the European Union, that essentially wants to create the human brain *in silico*, we are dreaming bigger than ever before. I have no doubt that the advances in the 21st century will be astounding. However, we are still just beginning this quest. Compared to other organ systems very little is

known about the human brain. I propose that the study of basic physiological processes, like breathing, is an excellent place to begin to fully understand the brain's function. The respiratory control system of the pons and medulla contains many of the hallmark characteristics of neural circuits; including gain control, conditioning, spontaneous and oscillatory components, neurmodulation and plasticity. Furthermore, whereas other brain phenomena like anxiety, learning and memory, or compassion can be difficult to measure; respiratory output is easily measured and quantified, meaning greater certainty in results. In summary, while self-awareness and memory are exciting brain functions in need of study, let us not forget that homeostatic behaviors, like breathing, offer a chance to study the complexity of neural circuits, with more quantitative behavioral readouts.

Chapter 2 Background and Literature Review

CENTRAL RESPIRATORY CHEMORECEPTION

Ventilation (also referred to herein as respiration or breathing) is the process an organism goes through to achieve adequate gas exchange. For animals the main function of ventilation is to absorb the molecular substrate for cellular respiration (i.e. O_2) and release the byproduct (i.e. CO_2). In the vast majority of mammals this is called "breathing" and is accomplished through the epithelium of the lungs. Air is moved into the lungs via negative pressure produced by contraction of the diaphragm muscle, and expelled passively when the diaphragm relaxes. Thus breathing requires alternating periods of diaphragm contraction and relaxation, and this continues, relatively uninterrupted, from birth until death.

Unlike the heart, the lungs have no intrinsic pacemaker cells. The rhythm of diaphragm contraction is controlled from the central nervous system. It is commonly accepted that the mammalian respiratory pattern generator (rCPG) is located in the Prebotzinger complex of the ventrolateral medulla (Smith, Ellenberger et al. 1991). This nucleus contains inspiratory and pre-inspiratory neurons that project, polysynaptically via premotor neurons, to respiratory motor neurons. Phrenic motor neurons receive indirect Prebotzinger input, causing them to oscillate between periods of high activity and quiescence. Thus their synaptic connections to the diaphragm translate the activity of the rCPG into physical ventilatory motor activity that we call breathing.

Respiratory rhythm is labile; increasing or decreasing depending on the metabolic demand. There are many homeostatic mechanisms that exist to match ventilation to metabolic demand. In the lungs there are mechano-, and irritant-, receptors that respond to lung stretch and irritants, respectively, and feedback via the brainstem to alter the respiratory rhythm (Pisarri, Jonzon et al. 1990). In the peripheral vasculature there are specialized organs (i.e. the aortic and carotid bodies) that sense changes in blood gases and also feedback to the brainstem to adjust the respiratory rhythm (O'Regan, Majcherczyk 1982). Finally, the respiratory centers in the brainstem receive input from higher brain centers, including the pons,

hypothalamus and cortex which drive breathing, and are thought to coordinate breathing changes needed for subconscious activities like the fear response and higher order processes like talking or singing.

Respiratory chemosensation (hereafter referred to as simply "chemoreception"), the sensation and regulation of CO_2/O_2 levels in the body, is a major homeostatic mechanism of respiration. Disruption of chemoreception is thought to result in a number of breathing disorders, including central congenital hypoventilation syndrome (CCHS) and central sleep apnea (Weese-Mayer, Berry-Kravis et al. 2003, Collop 2009). Initially it was thought that the cellular components of chemoreception resided solely in the central nervous system (O'Regan, Majcherczyk 1982). It was subsequently found that the carotid and aortic bodies (now known as peripheral chemoreceptors), were capable of mediated respiratory responses to altered O_2/CO_2 levels (Heymans, Bouckaert 1930). However, it was also observed that CO_2 chemoreception persisted even in the absence of functional carotid and aortic bodies (O'Regan, Majcherczyk 1982). The phenomenon of central CO_2 chemoreception was later confirmed in the 1950's when Leusen and colleagues used the ventricle perfusion technique to selectively reduce pH in the central nervous system, and observed increased ventilation (Leusen 1954). Thus it is understood that there are both central and peripheral chemoreceptors, the former being the focus of this thesis.

Probably because of anatomical distinction, carotid and aortic bodies were identified as peripheral chemoreceptors in the early part of the 20^{th} century, which won Corneille Heymans the Nobel Prize in 1938. On the other hand, the identity of central chemoreceptors was, and to a certain extent remains, elusive (Guyenet, Bayliss et al. 2009, Nattie, Li 2008). In the 1960's Loeschcke and colleagues began to search for central chemoreceptors in the ventral medulla. They found that acidic solution applied to certain areas of the ventral lateral medulla (VLM) increased ventilation (Mitchell, Loeschcke et al. 1963, Loeschcke 1982). These data led to the hypothesis that central chemoreceptor cells resided in particular regions of the VLM.

By the late 1980's the practice of brain slice electrophysiology to study neuroscience phenomena was in full swing, and the field of chemoreception was no exception. Finding brain regions that appeared to contribute to chemoreception was now not enough, and the new search was for chemoreceptor neurons. One of the first regions found to contain chemosensitive neurons was the nucleus of the solitary tract (NTS). The NTS is located in the dorsal medulla, opposite from chemosensitive regions (i.e. the VLM) identified decades before. In 1989, Millhorn and colleagues identified neurons in the commissural NTS (cNTS) that varied their action potential firing frequency in response to changes in CO_2/H^+ (Dean, Lawing et al. 1989). Most CO_2-sensitive neurons increased action potential firing when exposed to elevated CO_2 in the Ringers solution. It was also found that the CO_2-sensitivity was not dependent on synaptic input, indicating this was an intrinsic response of the neuron (Dean, Bayliss et al. 1990). To this day the neuronal cell type and CO_2 sensing mechanism in the NTS have eluded researchers.

The next region of the medulla found to house chemosensitive neurons was that of the medullary and midbrain raphe. The medullary raphe is the main grouping of serotonergic neurons in the hindbrain. In 1995 George Richerson observed CO_2/H^+-sensitive neurons in the medullary raphe, and identified them as serotonergic (Wang, Tiwari et al. 2001, Richerson 1995). Later, those of the midbrain were found to be CO_2-sensitive as well (Severson, Wang et al. 2003). In addition, the mechanism of CO_2 sensing was for the midbrain raphe chemosensitive neurons was found to be pH-sensitive TASK3 (twik-related acid-sensitive potassium channels isoform 3 or K2P9.1), although the ablation of TASK3 had no effect on whole animal CO_2 chemosensitivity (Mulkey, Talley et al. 2007), suggesting that although raphe neurons are chemosensitive, they are not major contributors to respiratory chemoreception. More recent studies, however, have provided evidence for a medullary serotonergic raphe neuronal role in central chemoreception. Dual use of Cre and Flpe receombinase systems have allowed for specific expression of DREADD receptors in rostral medullary raphe neurons (Armbruster, Li et al. 2007). When exposed to the exogenous ligand clozapine-*N*-oxide (CNO), DREADD (Designer Receptors Exclusively Activated by Designer Drugs) receptors hyperpolarize the cell membrane, thus silencing neuronal firing of action

potentials. Using this paradigm, the authors observed reduced CO_2 chemoreception *in vivo* when the rostral medullary serotonergic raphe neurons were inhibited (Ray, Corcoran et al. 2011).

In 2004 chemosensitive neurons were identified in a region that roughly corresponded to those identified by Loeschcke and colleagues in 1960's, as evident in the editorial "Back to the future: carbon dioxide cheomoreceptors in the mammalian brain" (Mitchell 2004). Mulkey and colleagues identified chemosensitive neurons in the rostral VLM, more specifically in a region of the brain called the retrotrapezoid nucleus, or RTN (Mulkey, Stornetta et al. 2004). The RTN was identified years before, in both cats and rats, as a region with major inputs into major respiratory centers of the medulla (Smith, Morrison et al. 1989, Pearce, Stornetta et al. 1989). In the study by Mulkey in 2004, and following work, the authors found that a subset of neurons in the RTN show increased action potential firing during elevated CO_2/H^+, and this is not due to synaptic, carotid body or respiratory CPG input (Mulkey, Stornetta et al. 2004). The chemosensitive RTN neurons are glutamatergic, project to major respiratory regions and express the transcription factor Phox2b (Mulkey, Stornetta et al. 2004, Stornetta, Moreira et al. 2006). Phox2b is a transcription fact that, when mutated in humans and mice, results in a near complete loss of chemoreception (Weese-Mayer, Berry-Kravis et al. 2003, Amiel, Dubreuil et al. 2009). In addition to dysfunctional chemoreception, mutations of Phox2b in mice resulted in the absence of chemoreceptor RTN neurons (Amiel, Dubreuil et al. 2009). More evidence of a role for RTN neurons in central chemoreception include; specific activation of Phox2b RTN neurons with channelrhodpsin2 results in cardio and ventilatory responses that are similar to those during hypercapnia, and when Phox2b RTN neurons are ablated using selective toxins decreased chemoreception is observed (Takakura, Moreira et al. 2008, Marina, Abdala et al. 2010, Abbott, Stornetta et al. 2009).

As apparent from above, there is range of evidence for a number of medullary regions and cell types (i.e. cNTS, medullary Raphe and RTN) to be involved in central chemoreception. The description here does not detail the work of Nattie and colleagues, who, using microdialysis of CO_2/H^+ into the medulla have determined a number regions (including those mentioned above) that appear to contribute to

chemoreception (Nattie, Li 2008). These observations have led to the "distributed theory" of central chemoreception, in which there are multiple neuronal groups that are sensitive to CO_2/H^+ and activate the respiratory system. The reason for multiple neuronal types with the same function is not clear, although the common response is "redundancy" (Nattie, Li 2008). Redundancy is the concept that important functions, like respiratory control, will have backups in case one becomes nonfunctional. This certainly appeals to logic. Yet, destruction of even a small number of these neurons in the Prebotzinger complex, the group of neurons necessary for respiratory rhythm to be generated (Tan, Janczewski et al. 2008), results in a loss of respiratory motor activity (Hayes, Wang et al. 2012). Even with a system so susceptible to small amounts of cell death breathing continues. An alternative view could be that each chemosensitive neuronal population contributes to central chemoreception in its own way. For instance, medullary raphe chemosensitive neurons could modulate behavior of the respiratory system (e.g. they are serotonergic and serotonin is a common neuromodulator), RTN neurons could provide more tonic excitatory input to the system (e.g. they are glutamtergic) and NTS neurons may effect peripheral autonomic reflexes (e.g. they are located in the major visceral relay center of the medulla). In any case, direct evidence to indicate a single chemoreceptor site is lacking. This would require specific ablation of the CO_2/H^+-sensing mechanism in a particular set of cells, while preserving all other function of those cells. This would likely be accomplished by using transgenic approaches to eliminate CO_2/H^+-sensing mechanisms in specific cell populations. To begin to construct such transgenic experiments we must first determine the cellular identity and sensing mechanisms of particular chemoreceptors cells. As such, in this thesis I have focused on determining CO_2/H^+-sensing mechanisms of RTN chemoreceptor neurons and astrocytes.

PURINERGIC SIGNALING

Adenosine tri-phosphate, ATP, a purine nucleotide, is an important player in molecular biology. Most notably ATP is the "molecular currency" of energy transfer within a cell. Much of the study of biochemistry is concerned with its synthesis and metabolism. ATP is generated during glycolysis,

photosynthesis and cellular respiration and the energy stored in the three phosphate bonds can be used for many cellular functions. For instance, transmembrane pumps, such as the Na-K-ATPase, use the energy stored in the phosphate bonds of ATP to move molecules against their electrochemical gradient. Intracellular signaling pathways utilize ATP as a substrate by kinases that phosphorylate proteins and lipids, and by adenylate cyclase, which produces the second messenger molecule, cyclic AMP. More recently ATP has been found to be involved in *inter*cellular signaling, acting in autocrine, paracrine and neurocrine mechanisms on membrane bound purinergic receptors (Burnstock 2007).

Perhaps due to the fact that ATP is such an important molecule for cell metabolism it was difficult for the scientific community to accept that it was involved in intercellular signaling. The first observation that ATP can act extracellularly occurred during experiments on cardiac tissue in 1929 (Drury, Szent-Gyorgyi 1929). Later it was found to act in the intestine and parts of the spinal cord (Buchthal, Engbaek 1947, Gillespie 1934). Exogenous ATP acts as an antagonist to certain anesthetics and is released from nerve axons (Kuperman, Okamoto et al. 1964, Kuperman, Volpert et al. 1964). In 1970 Geoffrey Burnstock postulated that ATP is used for specific intercellular signaling (Burnstock, Campbell et al. 1970). He discovered nerves in the autonomic nervous system innervating the gut and bladder that utilized ATP as their neurotransmitter. Since then ATP has been described as a neurotransmitter in the autonomic, central and nociceptive nervous systems and is a cotransmitter in many other regions (Burnstock 2007).

The membrane receptors whose ligands are purines and pyrimidines are commonly referred to as purinergic receptors. There are at least three different families of purinergic receptors; P1 (also referred to as adenosine receptors), P2X and P2Y receptors. Purinergic receptors have been found to be located in many regions of the body and function in an equally wide array of cellular processes.

P1 receptors are G-protein coupled receptors. There are four P1 receptor subtypes; A1, A2A, A2B and A3, which all respond to adenosine as their endogenous agonist. When A1 and A3 receptors are activated they stimulate G_s, increasing cAMP, whereas activation of A2A and A2B receptors stimulates G_i,

decreasing cAMP (Burnstock 2007). P1 receptor activation causes decreased heart rate, coronary vasodilatation and A2B, in combination with neutrin-1, is involved with axon elongation in neurons (Fredholm, IJzerman et al. 2011).

P2X receptors are ionotropic with two transmembrane domains. There are seven cloned P2X subtypes; P2X1-7, which all respond to ATP as their endogenous agonist. P2X receptors act as cation channels and upon activation by ATP, allow the flow of sodium, potassium and in some cases calcium. This causes depolarization of the cell membrane, which in turn can open Ca^{2+} channels (Dubyak, el-Moatassim 1993). P2X1-6 are all very similar in structure while P2X7 has an elongated intracellular peptide sequence on the carboxy end. When exposed to ATP it initially functions as the other P2X receptors, however if the ATP exposure is prolonged its pore enlarges and larger molecules can flow through. It is mainly expressed in immune and glial cells, and seems to allow the release of proinflamatory cytokines (Burnstock 2008). P2X receptors have been located in neurons, glia, bone, muscle, endothelium and hematopoietic cells (North 2002), and have functions including fast synaptic transmission (Bardoni, Goldstein et al. 1997), neurotransmitter release (Khakh, Henderson 1998) and pain signaling (Cockayne, Hamilton et al. 2000).

P2Y receptors are G-coupled with seven transmembrane spanning domains. There are eight P2Y receptor subtypes that have been cloned; P2Y1, P2Y2, P2Y4, P2Y6 and P2Y11 through P2Y14 (Erb, Liao et al. 2006, Burnstock 2007). P2Y receptors respond to a more varied array of endogenous agonists, including ATP, ADP and even uridine nucleotides UTP and UDP. The strength of response to these agonists varies by receptor subtype, which is sometimes used for subtype identification. For instance, if a cell exhibits a physiological response to ATP and UTP, but not ADP then the responsible receptor is likely either P2Y2 or P2Y4. G-proteins utilized by P2Y receptors are G_i, G_o, $G_{s,}$ G_q/G_{11}, $G_{\alpha i}$, G_{12} and G_i/G_o (Erb, Liao et al. 2006). Some P2Y receptor subtypes have specific G-proteins they associate with, but many can associate with multiple. For example, the P2Y2 receptor can activate G_o, G_q/G_{11} and G_{12} (Erb, Liao et al. 2006). Stimulation of these G-proteins by recombinant P2Y receptors can either activate phospholipase C (PLC) or adenylate cyclase, causing intracellular Ca^{2+} release or changes in cAMP levels, respectively. P2Y

signaling is responsible for numerous physiological responses (Burnstock 2007). P2Y receptors are thought to play a role in the activation of ion channels, including the volume-regulated anion channel (Darby, Kuzmiski et al. 2003) and of N-type Ca^{2+} channels (Abbracchio, Burnstock et al. 2006). P2Y receptor expression has been shown to influence cell proliferation in a number of cell types (Bagchi, Liao et al. 2005) and is upregulated in response to tissue injury (Ahn, Camden et al. 2000, Rohatgi, Sedehizade et al. 2003).

GLIAL CELLS

Glial cells were discovered during the mid-19th century, not too long after Scwhann and Schleiden theorized that all living matter was composed of cells in 1839 (Kimelberg 2004) It was Rudolf Virchow who first coined the term neuroglia, meaning "neural-cement," in 1856 (Ndubaku, de Bellard 2008). Virchow's histochemical stains illustrated clear distinctions between neurons and glia but were unable to delineate between the different subtypes of glia. Using the black chrome-silver reaction Camillo Golgi was the first to distinguish radial glia from other glia and neurons in 1895 (Kimelberg 2004). Ramon y Cajal was the first to use the term "astrocyte" t describe the fibrous and protoplasmic glial subset (Somjen 1988). In 1920, Cajal's student del Rio Hortega was able to distinguish oligodendrondrocytes and microglia from the other two glial subsets (Somjen 1988).

Glial cells have since been split into two broader classes of macroglia and microglia, the latter being the primary immune cell in the brain. Macroglia in the peripheral nervous system (PNS) consist of Schwann cells and satellite cells. Schwann cells' main role is that of axon myelination (Ndubaku, de Bellard 2008). Satellite cells line the exterior portion of the PNS and regulate the exterior chemical environment (Topilko 2007). Macroglia in the CNS consist of three distinct types; oligodendrocytes, polydendrocytes and astrocytes. Similar to Schwann cells, oligodendrocytes have small somas and large end feet that are responsible for axon myelination (Ndubaku, de Bellard 2008). Polydendrocytes, also called NG2 glia, are

the major proliferative cell type in the adult brain, mostly giving rise to new oligodendrocytes (Nishiyama, Komitova et al. 2009).

Astrocytes account for approximately 20-30% of brain cells in the mature CNS and are crucial for maintaining relative homeostasis (Hertz, Zielke 2004, Kimelberg 2004). In the microenvironment astrocytes mediate neurotransmitter and ionic homeostasis, synaptic formation, neuronal excitability and migration, detoxification and maintenance of the blood brain barrier (Markiewicz, Lukomska 2006). Astrocytes are thought to secrete a wide variety of neurotrophic factors, such as nerve growth factor, NGF, and brain-derived neurotrophic factor BDNF (Villegas, Poletta et al. 2003). Astrocytes regulate the extracellular space near synapses after high neuronal output by buffering potassium (Leis, Bekar et al. 2005, Olsen, Campbell et al. 2007) as well as neurotransmitters (Hertz, Zielke 2004).

Originally astrocytes, as well as other glial cells, were thought to simply provide neurons with structural support, but in more recent decades it has become clear that astrocytes also are directly involved in information processing. They are able to stabilize as well as modulate synaptic activity (Haydon, Blendy et al. 2009). Astrocytes express many neurotransmitter receptors that, when activated, initiate calcium signaling (Villegas, Poletta et al. 2003). These calcium waves spread between adjacent astrocytes via gap junctions and purinergic signaling (Inoue, Koizumi et al. 2007), which can then trigger release of ATP and glutamate; the main mediators of neuron-glia cross-talk (Villegas, Poletta et al. 2003). Astrocytes also have been found to communicate with microglia and capillaries (Inoue, Tsuda et al. 2007), making them a major playing or neurovascular coupling (Straub, Nelson 2007).

Chapter 3 Hypothesis and Specific Aims

Central chemoreception is the mechanism by which the brain regulates respiration in response to changes in tissue pH/CO_2. Chemosensitivity is particularly important during sleep and its disruption has been associated with central sleep apnea & hypoventilation syndrome (Weese-Mayer, Berry-Kravis et al. 2003, Collop 2009). A region of the brainstem called the retrotrapezoid nucleus (RTN) is thought to be an important site of central chemoreception (Mulkey, Stornetta et al. 2004).

Recent evidence suggests that RTN chemoreception involves two mechanisms. The first appears to be H^+-mediated activation of pH-sensitive neurons, via inhibition of TASK2 channels (Wang, Benamer et al. 2013). When TASK2 is ablated the CO_2/H^+-sensitivity of RTN neurons reduced, but not entirely ablated. The second sensing mechanism appears to involve paracrine release of ATP that acts on neuronal P2 receptors (Gourine, Llaudet et al. 2005). Hypercapnia (elevated CO_2/H^+) induces ATP release at the medullary ventral surface and pharmacological blockade of P2 receptors, or destruction of ATP by apyrase, in this region blunts central chemoreception *in vivo* (Gourine, Llaudet et al. 2005). It is thought that H^+/CO_2-evoked ATP release acts on P2 receptors of pH-sensitive RTN neurons. It has been postulated that astrocytes in the region release ATP, as they have been found to release ATP in response to various stimuli in other brain regions (Straub, Nelson 2007). However, the source and mechanisms of this purinergic drive to breathe remains undetermined.

Hypothesis. I hypothesize that RTN astrocytes sense CO_2/H^+ by inhibition of Kir4.1/Kir5.1 channels, which results in membrane depolarization. I also hypothesize that ATP released in response to CO_2/H^+ acts on P2-receptors expressed by RTN neurons to increase neuronal action potential firing, and that this purinergic mechanism of chemoreception is restricted to the ventral surface, affecting primarily RTN neurons.

*Sp*ecific Aim 1. Determine the CO_2/H^+-sensitive membrane properties of glial cells in the RTN. Subsequently, identify the cell type (i.e. astrocyte, microglia etc.) and sensing mechanism (i.e. channel). This was accomplished via patch-clamp recordings from glial cells, and post-hoc immunohistochemical analysis of glial cell markers.

Specific Aim 2. Determine the role of P2 receptors on RTN neuronal chemosensitivity, in vivo and in vitro. For *in vitro* studies, this was accomplished by making loose patch recordings from rat brain slices of RTN neurons and measuring their response to CO_2/H^+ under control conditions and in the presence of P2 antagonists. Also, I will use neuronal recordings as an assay to determine the mechanism of release by using pharmacology to block gap junction hemichannels and inhibitors of vesicle fusion. *In vivo*, the effect of P2 antagonist injection into the RTN on cardiorespiratory parameters was studied in anesthetized adult rats.

Specific Aim 3. Determine the regional specificity of puringergic signaling contribution to central chemoreception in vivo and in vitro. This was accomplished using the same methods as described in Aim 2. *In vitro*, loose patch recordings were made from CO_2-sensitive neurons in the caudal NTS (cNTS) and medullary raphe pallidus (RPa). These cells were assayed to see if they functionally express P2 receptors, and if these receptors contribute to their chemosensitivity. Similarly, *in vivo*, cardiorespiratory responses to hypercapnia in anesthetized rats were tested under control conditions and during P2 receptor blockade in the cNTS and RPa. In addition, ATP injection into these regions was performed to test for functional P2 receptor expression *in vivo*.

Chapter 4 Astrocyte of the Retrotrapezoid Nucleus Sense H+ by Inhibition of a Kir4.1-Kir5.1-like Current and May Contribute to Chemoreception by a Purinergic Mechanism

INTRODUCTION

A region of the caudal brain stem called the retrotrapezoid nucleus (RTN) contains a population of neurons that are highly pH sensitive in vivo and in vitro, are glutamatergic, and project to respiratory centers to influence breathing (Abbott, Stornetta et al. 2009, Mulkey, Stornetta et al. 2004). The mechanism by which these RTN chemoreceptors sense pH involves inhibition of an unidentified voltage-independent K^+ conductance (Mulkey, Stornetta et al. 2004). The absence of an identified molecular pH sensor in RTN neurons has raised the possibility that multiple mechanisms contribute to chemoreception (Filosa, Putnam 2003) and, despite the apparent intrinsic pH sensitivity of RTN neurons, it remains possible that local paracrine mechanisms contribute to chemoreception by the RTN.

There is increasing evidence that adenosine 5′-triphosphate (ATP), released by astrocytes, is an important mediator of chemoreception. Previous in vivo studies showed that hypercapnia evoked the discrete release of ATP within the RTN (Gourine, Llaudet et al. 2005). Further, application of ATP into the RTN stimulated respiratory output, whereas application of an ATP receptor antagonist (pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate [PPADS]) to the RTN lowered CO_2 respiratory responses (Gourine, Llaudet et al. 2005). Recently, it was shown that RTN astrocytes respond to bath acidification with Ca^{2+}-dependent, exocytosis-mediated, ATP release (Gourine, Kasymov et al. 2010). In addition, photostimulation of RTN astrocytes expressing channelrhodopsin-2 (ChR2) resulted in a robust increase in breathing (Gourine, Kasymov et al. 2010), strongly suggesting that astrocytes contribute to respiratory drive. This study also provided evidence suggesting that ATP release from astrocytes is a requisite signal for neuronal pH sensitivity (Gourine, Kasymov et al. 2010). At the cellular level, we have shown that purinergic signaling can activate pH-sensitive RTN neurons via P2Y receptors (Mulkey, Mistry et al. 2006). However, we also found that the pH sensitivity of RTN neurons in HCO_3^--free HEPES-buffered

media was retained when ATP receptors were blocked with PPADS (100 μM) (Mulkey, Mistry et al. 2006), indicating that ATP is not a requisite component of neuronal pH sensitivity. These results suggest that RTN neurons are intrinsically pH sensitive but that CO_2-evoked release of ATP can modulate the activity of pH-sensitive neurons and contribute to the integrated output of the RTN (i.e., respiratory drive) during hypercapnia.

The goals of this study were to *1*) confirm that RTN astrocytes are pH sensitive, *2*) identify mechanism(s) by which these cells sense changes in pH, and *3*) determine the extent to which they contribute to chemoreception. To make these determinations, we used the brain slice preparation and a combination of patch-clamp electrophysiology and immunohistochemistry to identity pH-sensitive glia and to determine the mechanism by which they sense changes in pH. To explore the possibility that pH-sensitive RTN astrocytes stimulate activity of pH-sensitive neurons by a purinergic mechanism, we determined the effect of fluorocitrate-mediated astrocyte activation on pH-sensitive neurons. We found that pH-sensitive RTN glia are protoplasmic astrocytes that sense H^+, in part, by inhibition of a Kir4.1–Kir5.1-like current and may contribute to respiratory drive by increasing activity of pH-sensitive RTN neurons by a purinergic-dependent mechanism.

METHODS

Brain stem slices

Slices containing the RTN were prepared as previously described (Jiang, Xu et al. 2001, Matthias, Kirchhoff et al. 2003). Briefly, neonatal rats (7–12 days postnatal) were decapitated under ketamine/xylazine anesthesia and transverse brain stem slices (300 μm) were cut using a microslicer (DSK 1500E; Dosaka, Kyoto, Japan) in ice-cold substituted Ringer solution containing (in mM): 260 sucrose, 3 KCl, 5 $MgCl_2$, 1 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose, and 1 kynurenic acid. Slices were incubated for about 30 min at 37°C and subsequently at room temperature in normal Ringer solution

(in mM): 130 NaCl, 3 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 10 glucose. Both substituted and normal Ringer solutions were bubbled with 95% O_2-5% CO_2.

Electrophysiology

Individual slices were transferred to a recording chamber mounted on a fixed-stage microscope (Zeiss Axioskop FS) and perfused continuously (~2 ml min^{-1}) with a bath solution composed of (in mM): 140 NaCl, 3 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 *N*-2-hydroxyethylpiperazin-*N*′-2-ethanesulfonic acid (HEPES), 10 glucose; pH was adjusted between 6.9 and 7.5 by addition of HCl or NaOH. To test pH sensitivity of RTN astrocytes in bicarbonate-based buffer slices were perfused with normal Ringer solution bubbled with 95% O_2-5% CO_2 (bath pH = 7.45). The pH of the bicarbonate-based bath solution was decreased to a pH of about 6.8 by bubbling with 15% CO_2 as previously described (Filosa, Putnam 2003).

All recordings were made with an Axopatch 200B patch-clamp amplifier, digitized with a Digidata 1322A A/D converter, and recorded using pCLAMP 10.0 software (Molecular Devices). Recordings were obtained at room temperature with patch electrodes pulled from borosilicate glass capillaries (Warner Instruments) on a two-stage puller (P89; Sutter Instrument) to a DC resistance of 4–7 MΩ when filled with an internal solution containing the following (in mM): 120 KCH_3SO_3, 4 NaCl, 1 $MgCl_2$, 0.5 $CaCl_2$, 10 HEPES, 10 EGTA, 3 Mg-ATP, 0.2% biocytin, and 0.3 GTP-Tris (pH 7.2); electrode tips were coated with Sylgard 184 (Dow Corning). Voltage-clamp recordings from glia were made at a holding potential of −80 mV and in the presence of tetrodotoxin (TTX, 0.1 μM) to block neuronal activity. Holding current, conductance, and current–voltage (*I–V*) relationships were determined using voltage steps between −40 and −150 mV. Neuronal firing rate histograms were generated by integrating action potential discharge in 10-s bins and plotted using Spike 5.0 software. It is important to recognize that in a whole cell recording circuit, recording cells with a low membrane resistance (e.g., passive astrocytes) in series with a large access resistance (R_a) can result in voltage-clamping error. In our experiments, we limited R_a to about 10 MΩ by preparing tissue from young animals; capacitance and R_a compensation (70%) were used to

minimize voltage errors as previously described (Matthias, Kirchhoff et al. 2003). Recordings were discarded if R_a varied >10% during an experiment. A liquid junction potential of 10 mV was corrected off-line. Data were reported as means ± SE and were analyzed by the paired *t*-test or ANOVA and Dunnett's multiple comparison test when appropriate ($P < 0.05$).

Immunohistochemistry

Recorded slices were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 2 h at 4°C and rinsed in phosphate-buffered saline (PBS). Slices were blocked and permeabilized in PBS containing 5% normal goat serum and 0.4% Triton X-100 for 1 h at room temperature. Biocytin was detected by incubation in Alexa 488–conjugated strepavidin (1:1,000; Invitrogen) for 1 h at room temperature. After three PBS washes, the slices were incubated in primary antibodies overnight at 4°C, washed, and then incubated in secondary antibodies for 1 h at room temperature. Slices were washed in PBS and mounted in Vectashield. Images were collected on a Leica TCS SP2 confocal microscope equipped with 488-, 543-, and 633-nm laser lines and tunable emission wavelength detection. For each labeled slice, a biocytin-positive cell was identified and confocal *z*-stacks were collected sequentially for the other two channels to detect glial antigens. A cell was scored as immunopositive if the immunoreactivity for one or more glial markers was detected in the biocytin-positive cell body and processes. To determine background fluorescence of the tissue and nonspecific binding of secondary antibodies, we tested secondary antibodies without preincubation in primary antibody. These methods have been optimized for 300-μm slices sequentially detecting biocytin and glial antigens.

Drugs

Tetrodotoxin was purchased from Alomone Labs (Bethlehem, Israel); all other chemicals were obtained from Sigma (St. Louis, MO). Fluorocitrate was prepared as previously described (Holleran, Babbie et al. 2001). Briefly, the Ba^{2+} salt of fluorocitric acid was dissolved in 0.1 M HCl and the Ba^{2+} was precipitated by the addition of Na_2SO_4 (0.1 M). This solution was centrifuged at 800 *g* for 10 min and the supernatant

removed and diluted in HEPES-buffered medium to a final concentration of 100 μM (pH 7.3 by addition of 1 N NaOH). To confirm that Ba^{2+} was adequately removed during the preparation of fluorocitrate, we also prepared a solution of equal Ba^{2+} concentration minus fluorocitric acid and subjected it to the same precipitation protocol described earlier.

RESULTS

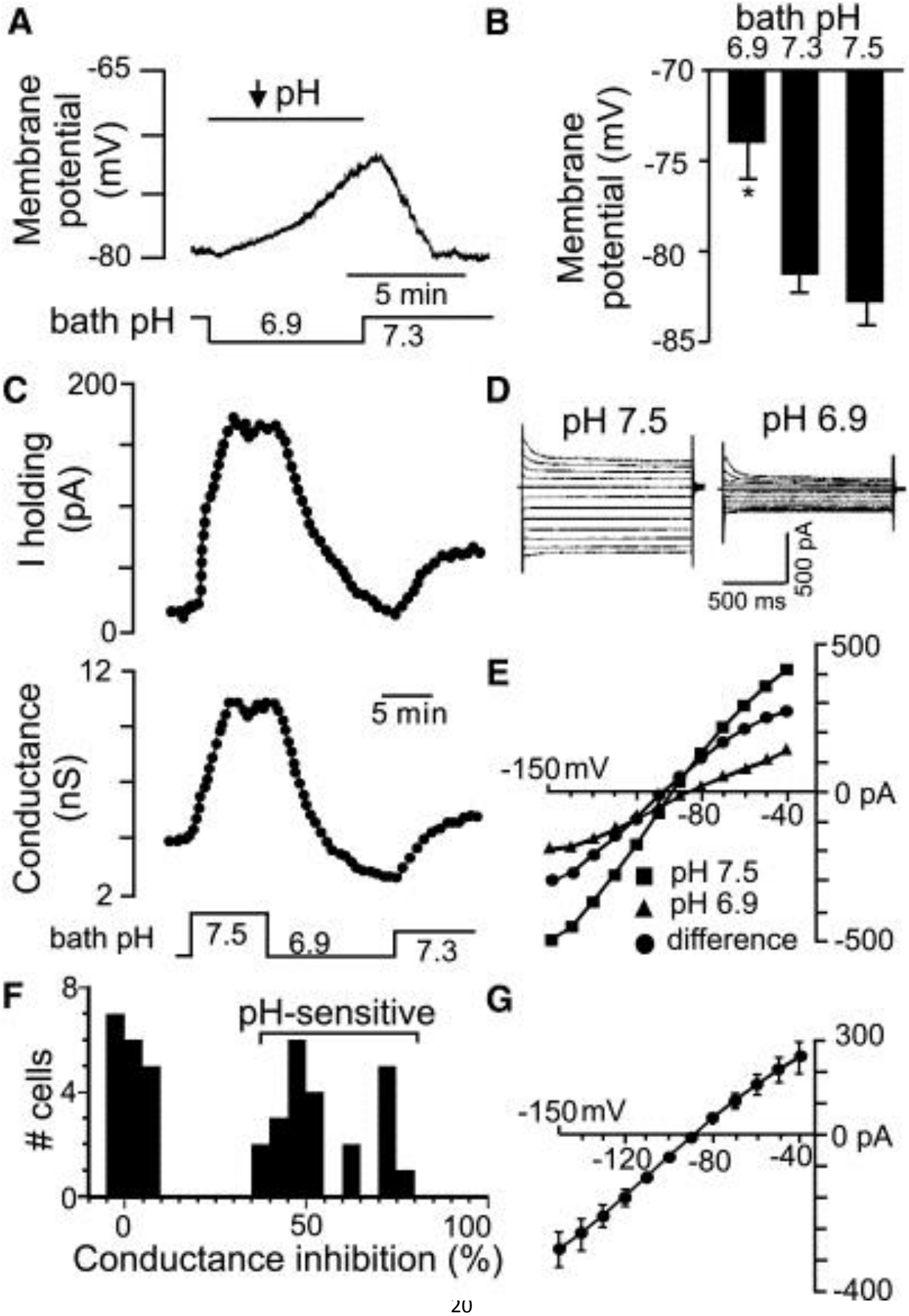
This study consists of two series of experiments. First, I made whole cell recordings to identify pH-sensitive RTN glia and to characterize the mechanism by which they sense pH. I initially identified glia by their small size, hyperpolarized membrane potential, and inability to form action potentials during depolarizing current injection; cellular identity was later confirmed using immunohistochemistry. In a second series of experiments, I made loose-patch recordings from pH-sensitive RTN neurons to determine the effects of fluorocitrate-mediated astrocyte activation on baseline activity and pH sensitivity of RTN neurons and to test effects of P2-receptor antagonists on neuronal CO_2 sensitivity.

Astrocyte chemoreceptors

Previous sharp electrode recordings showed that ≤44% of RTN glia respond to extracellular acidification with membrane depolarization (Fukuda, Honda et al. 1978). In the whole cell configuration and HCO_3^--free HEPES-buffered solution we found that roughly 20% of RTN glia were pH sensitive; cells were considered pH sensitive if they showed >10 mV/pH unit change in membrane potential, ≥40 pA change in holding current, or 35% change in conductance when cycling bath pH between 7.5 and 6.9. Based on these criteria, we found a bimodal distribution of pH-sensitive and -insensitive glial cells (Fig. 1*F*). In current-clamp, pH-sensitive RTN glia have a resting membrane potential of −80 ± 1 mV (n = 8) and respond to acidification from pH 7.5 to 6.9, with a depolarization of 8.8 ± 1.4 mV (n = 8) that occurs within about 6 min (Fig. 1, *A* and *B*). We hypothesize that the slow kinetics of pH-sensitive astrocytes is reflective of their modulatory role and likely helps maintain stable chemoreceptor output despite fast

Figure 1

Characteristics of pH-sensitive retrotrapezoid nucleus (RTN) glia in HEPES buffer. *A*: traces of membrane potential and bath pH show that acidification from 7.3 to 6.9 causes a reversible depolarization. *B*: summary of H^+ effects on membrane potential of pH-sensitive astrocytes ($n = 5$, $^*P < 0.05$). *C*: traces of holding current (*top*) and conductance (*bottom*) during changes in bath pH show that alkalization increases and acidification decreases outward current and conductance. *D* and *E*: current responses to voltage steps from −80 mV to between −40 and −150 mV (*E*) and corresponding current–voltage (*I–V*) relationships under alkaline and acidic conditions (*D*). To isolate the pH-sensitive *I–V* (♣), we subtract *I–V* relationships obtained at pH 6.9 (▲) from those recorded at pH 7.5 (■). *F*: percentage change in conductance during acidification from pH 7.5 to 6.9 observed from all glial cells recorded in the RTN. *G*: average *I–V* relationship ($n = 5$) of pH-sensitive current reverses near the equilibrium potential for K^+ (E_{K^+}, arrow).

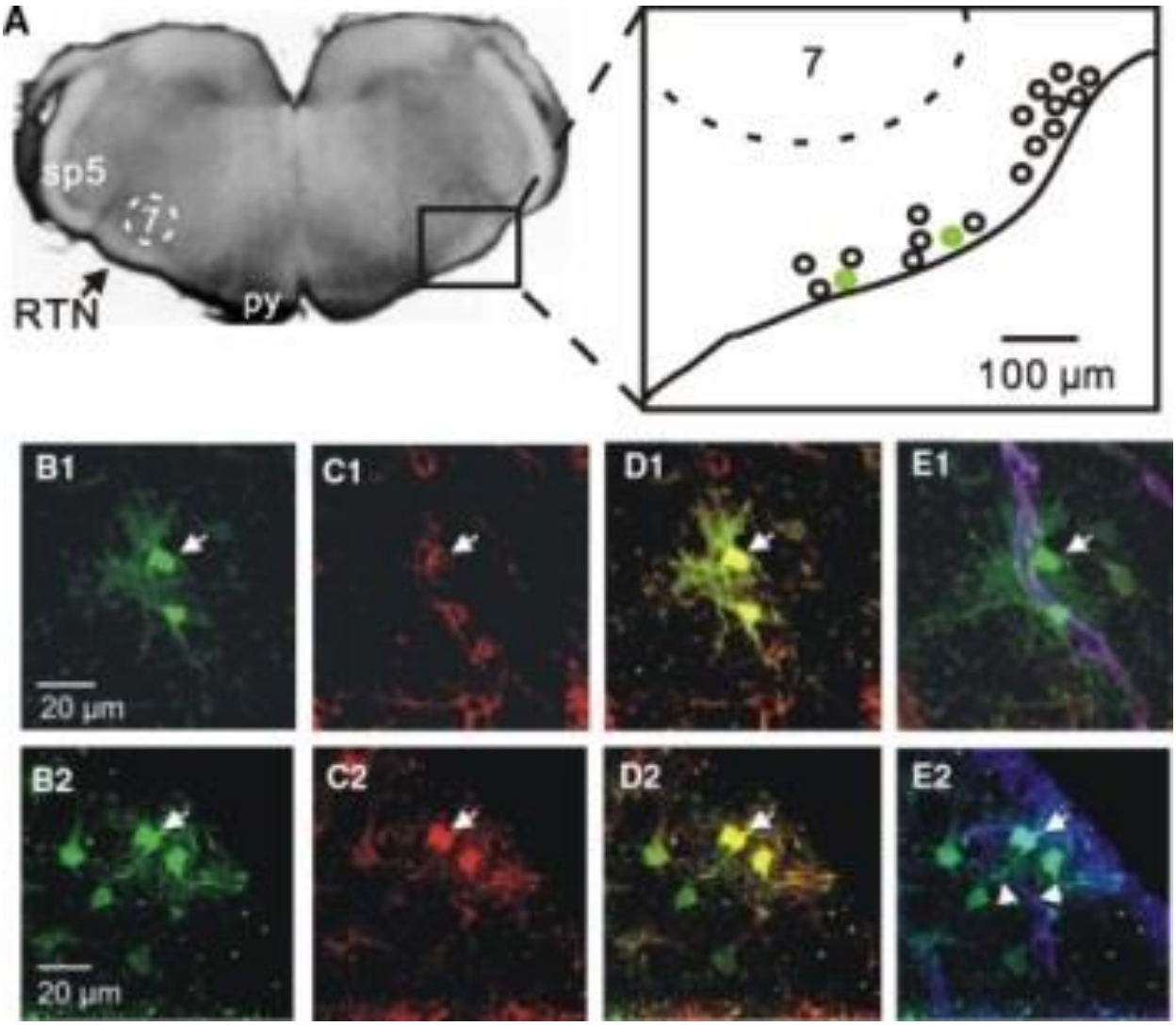

A
Membrane potential (mV)
-65
-80
pH
5 min
bath pH
6.9
7.3
B
bath pH
6.9 7.3 7.5
-70
-75
-80
-85
Membrane potential (mV)
*
C
I holding (pA)
200
0
Conductance (nS)
12
2
5 min
bath pH
7.5
6.9
7.3
D
pH 7.5
pH 6.9
500 pA
500 ms
E
500
-500
-150 mV
-80
-40
0 pA
pH 7.5
pH 6.9
difference
F
cells
8
4
0
pH-sensitive
0
50
100
Conductance inhibition (%)
G
300
-400
-150 mV
-120
-80
-40
0 pA

breath-by-breath oscillations in tissue pH (Millhorn, Eldridge et al. 1984). In contrast, pH-insensitive glia have a resting membrane potential of −74 ± 3 mV (n = 12) that did not vary over the same pH range. After recording, cells were filled with biocytin to characterize morphology and immunohistochemical phenotype. We found that 18 of 20 (90%) pH-sensitive glia were immunoreactive for aldehyde dehydrogenase 1L1 (Aldh1L1) (Fig. 2, *A*–*C*), an antigen that is expressed by protoplasmic astrocytes (Cahoy, Emery et al. 2008). In addition, pH-sensitive glia typically extended numerous fine radial projections near blood vessels (Fig. 2*D*) and to be dye-coupled (evidence of gap junctions) to other astrocytes (Fig. 2, *A*–*C*). These results support recent evidence that GFAP-expressing cells near the ventral surface are CO_2 sensitive (Gourine, Kasymov et al. 2010) and indicate that pH-sensitive glia are protoplasmic astrocytes.

To further characterize electrical properties of pH-sensitive astrocytes and establish their role as intrinsic pH sensors, we made voltage-clamp recordings of whole cell currents in HEPES buffer at control pH 7.3 and during exposure to alkaline and acidic conditions. Note that these experiments were performed in TTX (0.1 μM) to block neuronal activity. Under control conditions, pH-sensitive astrocytes display high K^+ conductance and exhibit fairly time- and voltage-independent responses to voltage steps. These properties are consistent with those of passive astrocytes described in the hippocampus (Matthias, Kirchhoff et al. 2003, Zhou, Schools et al. 2006) and brain stem (Grass, Pawlowski et al. 2004). Astrocytes in the RTN respond to acidification from pH 7.5 to 6.9, with a decrease in holding current of 91.8 ± 10 pA (n = 23) and a decrease in conductance of 6.8 ± 0.8 nS (n = 23) (Fig. 1*C*). This corresponds with a decrease in whole cell current of about 53% at +40 mV. By comparison, RTN astrocytes are more pH sensitive than astrocytes in a nonrespiratory region like the hippocampus; passive hippocampal astrocytes respond to acidification from 7.4 to 6.0 with a decrease in whole cell current at +20 mV of only about 28% (Zhou, Xu et al. 2009). To confirm that the pH sensitivity of RTN astrocytes is determined by intrinsic membrane properties, rather than intercellular current spread through gap junctions, we tested the effects of carbenoxolone (a nonspecific gap junction blocker) on astrocyte pH sensitivity. Carbenoxolone

Figure 2

pH-sensitive RTN glia are gap junction coupled protoplasmic astrocytes. *A, left*: a transverse slice between bregma −11.6 to −11.2 mm that contains the RTN. *Right*: map showing relative locations of 18 pH-sensitive RTN astrocytes (green circles correspond to the cells shown in the bottom panels). *B1* and *B2*: biocytin filled pH-sensitive RTN glial cells (green, arrows) and dye-coupled cells (other green cells). *C1* and *C2*: aldehyde dehydrogenase 1L1-labeled (Aldh1L1, red) astrocytes. *D1* and *D2*: merged images of the same cells in *B* and *C* show double-labeling for biocytin (green) and Aldh1L1 (red); the pH-sensitive astrocytes (arrows) and dye-coupled partners colocalize with Aldh1L1 labeling. *E1* and *E2*: merged images of the same cells in *B* and *C* show double-labeling for biocytin (green) and laminin (blood vessels, blue). Note that pH-sensitive RTN astrocytes have cell bodies positioned near blood vessels (*E1*) and extend processes to blood vessels (truncated arrows in *E2*). Each image is the average projection of a confocal stack; around 30 images taken every 0.26 μm in the *Z* plane.

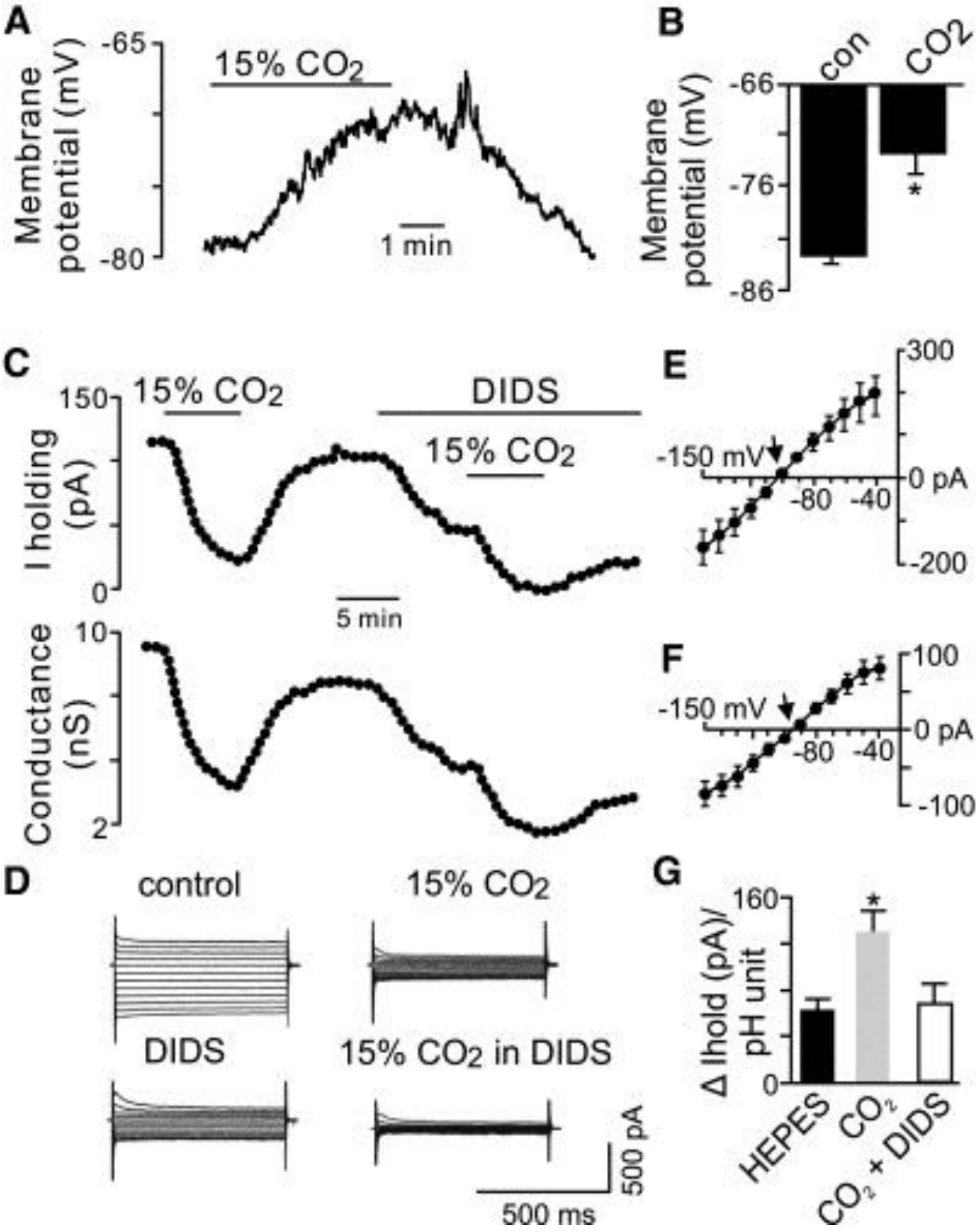

A
sp5
7
RTN
py
100 µm
B1
C1
D1
E1
20 µm
B2
C2
D2
E2
20 µm

(100 μM) increased the pH-sensitive current by roughly 18% (Data not shown), suggesting that RTN astrocytes are intrinsically pH sensitive. Astrocytes are known to express high levels of the electrogenic Na^+/HCO_3^- cotransporter (NBC), which can generate an inward depolarizing current (i.e., HCO_3^- efflux) during bath acidification (Deitmer 1991, Munsch, Deitmer 1994). To determine whether activity of the NBC contributes to the pH-sensitive current in RTN astrocytes, we characterized CO_2/H^+ sensitivity of RTN astrocytes in normal Ringer solution (26 mM HCO_3^-, 5% CO_2). In current-clamp, pH-sensitive RTN astrocytes had a membrane potential of −82 ± 1.2 mV (n = 3), which is about 2 mV hyperpolarized to resting potential of astrocytes in HCO_3^--free HEPES buffer, and responded to hypercapnia (15% CO_2, pHo = 6.8) with a membrane depolarization of 9 ± 2.3 mV (Fig. 3, *A* and *B*). In voltage-clamp, exposure to 15% CO_2 decreased holding current from 139.8 ± 20 to 54.9 ± 8.9 pA (n = 8), which corresponded with a change in holding current of 130 ± 17 pA/pH unit, whereas in HEPES buffer acidification from 7.3 to 6.9 changed in the holding current 62.7 ± 6 pA/pH unit (Fig. 3*G*). In addition, the CO_2/H^+-sensitive current had a reversal potential of −105 mV (Fig. 3*E*), which is more negative than the reversal potential of the pH-sensitive current in HCO_3^--free HEPES buffer, suggesting that activity of one or more HCO_3^- transporters contributes to pH sensitivity of RTN astrocytes. The most likely candidate is the electrogenic NBC, which can generate a depolarizing inward current (i.e., HCO_3^- efflux) during bath acidification (Chesler 2003, Deitmer 1991). Therefore we used diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS, an NBC blocker) to isolate the contribution of this transporter to pH sensitivity of RTN astrocytes. At control pH = 7.4, exposure to DIDS (0.5 mM) decreased holding current by 41.6 ± 8 pA (n = 3) (Fig. 3*C*). In the continued presence of DIDS, exposure to 15% CO_2 decreased holding current by 45.2 ± 13 pA (n = 3) (Fig. 3*C*), which corresponds with a change in holding current of 69.5 ± 20 pA/pH unit (Fig. 3*G*). The CO_2/H^+-sensitive current in DIDS reversed at −94 mV (i.e., near the equilibrium potential for K^+ [$E_K{}^+$]) (Fig. 3, *D* and *F*), as expected for a current dominated by K^+ conductance.

The DIDS-sensitive current exhibited a reversal potential of −104 mV, which is slightly hyperpolarized to

Figure 3

Characteristics of pH-sensitive RTN glia in bicarbonate buffer. *A*: traces of membrane potential shows that exposure to 15% CO_2 causes a reversible membrane depolarization. *B*: summary of the effects of 15% CO_2 on membrane potential pH-sensitive astrocytes (n = 3). *C*: trace of holding current shows that 15% CO_2 decreases outward current and conductance. Under control conditions (i.e., 5% CO_2, pHo = 7.45), exposure to diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS, 0.5 mM) also decreased holding current close to 100 pA. In the continued presence of DIDS, a second exposure to 15% CO_2 decreased holding current also by about 100 pA. *D* and *E*: current responses to voltage steps from −80 mV to between −40 and −150 mV during 5% and 15% CO_2 exposure in bicarbonate buffer alone or in the presence of DIDS (*D*) and corresponding *I–V* relationships of the average CO_2/H^+-sensitive current (i.e., difference current) (n = 3) in bicarbonate buffer alone (*E*) or bicarbonate buffer with DIDS (*F*). Note that under control conditions the reversal potential of the CO_2/H^+-sensitive current is hyperpolarized to E_{K^+} (arrow in *E*), but when activity of the electrogenic Na^+/HCO_3^- cotransporter (NBC) is blocked with DIDS, the CO_2/H^+-sensitive current reverses near E_{K^+} (arrow in *F*). *G*: summary data comparing effects of CO_2/H^+ on holding current (at a holding potential of −80 mV) in HEPES and bicarbonate-buffered media. Activity of the NBC significantly increased acid-induced changes in holding current compared with HCO_3^--free HEPES solution or in the presence of DIDS (*P < 0.05).


A
Membrane potential (mV)
-65
-80
15% CO2
1 min
B
Membrane potential (mV)
-66
-76
-86
con
CO2
*
C
I holding (pA)
150
0
15% CO2
DIDS
15% CO2
5 min
Conductance (nS)
10
2
D
control
15% CO2
DIDS
15% CO2 in DIDS
500 pA
500 ms
E
300
-150 mV
0 pA
-80
-40
-200
F
100
-150 mV
0 pA
-80
-40
-100
G
Δ Ihold (pA)/ pH unit
160
0
*
HEPES
CO2
CO2 + DIDS

the predicted reversal potential for the NBC (E_{NBC}). For example, the E_{NBC} was calculated with the following equation (Newman 1991): $E_{NBC} = RT/F(n - 1) \ln [Na^+]_i[HCO_3^-]_i^n/[Na^+]_o[HCO_3^-]_o^n$, where n is the $2HCO_3^-$:$1Na^+$ stoichiometry and R (gas constant), T (temperature), and F (Faraday constant) have their usual meanings. For a temperature of 22°C, $[Na^+]_o$ = 157 mM, $[Na^+]_i$ = 4 mM, $[HCO_3^-]_o$ = 26 mM, and $[HCO_3^-]_i$ was estimated to be 22 mM using the Henderson–Hasselbalch equation as previously described(Gross, Hawkins et al. 2001); we calculated E_{NBC} to be −99 mV. It is not clear why the DIDS-sensitive current reversed about 5 mV negative to E_{NBC}, although DIDS has been shown to have nonspecific effects on anion exchangers and Cl^- channels in glia(Munsch, Deitmer 1994), which may account for the discrepancy. To confirm that Cl^- channels do not contribute to CO_2 sensitivity of RTN astrocytes, we tested CO_2 sensitivity in low chloride (50 mM) buffer.

The low Cl^- solution was made isoosmotic by replacing 80 mM NaCl with an equal amount of Na-gluconate. We found the CO_2/H^+-sensitive current was not affected by chloride reduction. For example, there was no difference between the CO_2/H^+-induced change in holding current under control conditions (92.3 ± 38 pA; n = 3) and in low Cl^- buffer (91.3 ± 37 pA; n = 3) (not shown). Likewise, amplitude and reversal potential of the CO_2/H^+-sensitive current were similar in control and low Cl^- conditions (data not shown).

In the absence of NBC activity (HCO_3^--free HEPES buffer or Ringer solution with DIDS), the CO_2/H^+-sensitive current expressed by RTN astrocytes reversed near the equilibrium potential for K^+ (E_K^+ = −97 mV), suggesting that one or more pH-sensitive K^+ channels contribute to the pH sensitivity of RTN astrocytes. Astrocytes have been shown to express several types of pH-sensitive K^+ channels, including background TASK channels and heteromeric Kir4.1–Kir5.1 channels. In particular, heteromeric Kir4.1–Kir5.1 channels are exquisitely sensitive to pH in the physiological range (Casamassima, D'Adamo et al. 2003, Xu, Yang et al. 2000), are preferentially expressed by astrocytes (Hibino, Fujita et al. 2004, Tang, Taniguchi et al. 2009), and in situ hybridization shows detectable levels of both Kir4.1 and Kir5.1 mRNA

in the RTN (Wu, Xu et al. 2004). Therefore we consider heteromeric Kir4.1–Kir5.1 channels the most likely molecular pH sensor in RTN astrocytes.

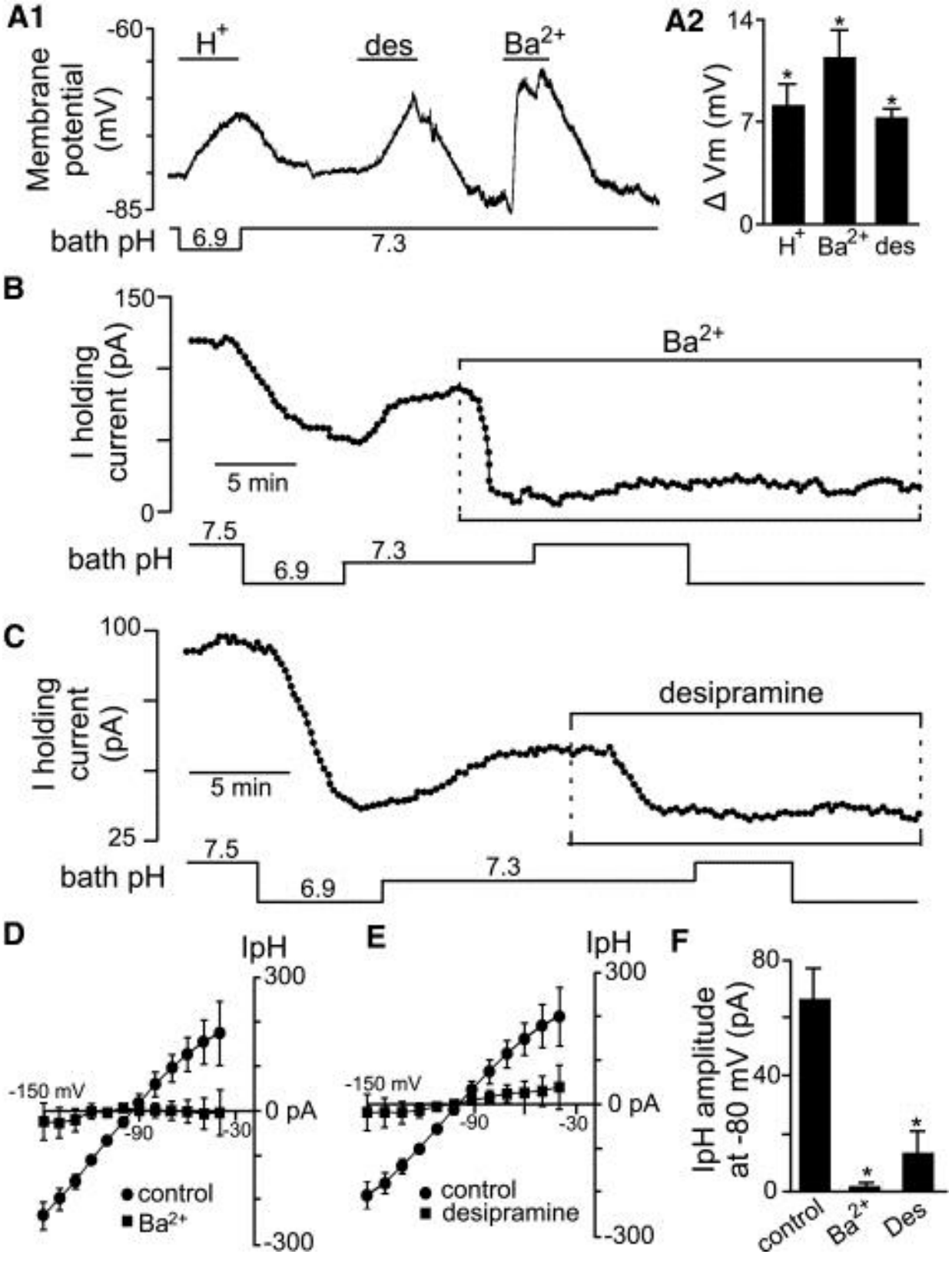
Heteromeric Kir4.1–Kir5.1 channels confer pH sensitivity to RTN astrocytes

The possible contribution of Kir channels can easily be distinguished from background K^+ channels based on Ba^{2+} sensitivity (Olsen, Sontheimer 2008); most Kir channels, but not background K^+ channels, are fully inhibited by micromolar concentrations of Ba^{2+} (for review of background and Kir channel pharmacology see (Kubo, Adelman et al. 2005, Goldstein, Bayliss et al. 2005, Patel, Honore 2001). Therefore to determine whether heteromeric Kir4.1–Kir5.1 channels contribute to pH sensitivity in RTN astrocytes, we first determine effects of Kir channel inhibition by Ba^{2+} (100 μM) on membrane potential and the pH-sensitive current. In current-clamp, Ba^{2+} depolarized the membrane potential by 16.7 ± 3.0 mV (n = 4) (Fig. 4*A*). In voltage-clamp (at −80 mV, TTX), Ba^{2+} decreased outward current by 86.5 ± 5 pA (n = 4) (Fig. 4*B*) and blocked the pH-sensitive current by 96.5 ± 3.5% (n = 4) (Fig. 4, *B*, *D*, and *F*). Note that if a more Ba^{2+}-sensitive background channel like TWIK (Ba^{2+} half-maximal inhibitory concentration [IC_{50}] = 100 μM; (Patel, Honore 2001)) or TASK-1 (Ba^{2+} IC_{50} = 350 μM; (Patel, Honore 2001)) contributed to the pH-sensitive current in RTN astrocytes, we would expect that only a portion (at best ∼50%) of the pH-sensitive current would be blocked by 100 mM Ba^{2+}. However, we show that this concentration of Ba^{2+} virtually eliminated the pH-sensitive current, suggesting that background K^+ channels do not confer pH sensitivity to RTN astrocytes. Inhibition of other voltage-gated K^+ channels with tetraethylammonium (TEA, 10 mM) or 4-aminopyridine (4-AP, 50 μM) had no effect on the pH-sensitive current in RTN astrocytes (Supplemental Fig. S1*A*). These results indicate that one or more Kir channels contribute to the pH-sensitive current in RTN astrocytes.

To differentiate the contribution of Kir4.1–Kir5.1 channels from other Kir channels, we inhibited Kir4.1-containing channels (i.e., homomeric and heteromeric channels) with desipramine (100 μM). At this concentration, desipramine inhibits channels containing Kir4.1 subunits, with minimal effects on other

Figure 4

pH sensitivity of RTN glia involves heteromeric Kir4.1 channels. *A1*: membrane potential trace shows that inhibition of Kir channels mimics the effects of H^+; Ba^{2+} (100 μM) and desipramine (des, 100 μM) depolarize membrane potential by about 18 and 9 mV, respectively. *A2*: average depolarization of RTN pH-sensitive astrocyte when exposed to either H^+ (acidification from 7.3 to 6.9, $n = 5$), 100 μM Ba^{2+} ($n = 4$), or 100 μM desipramine ($n = 5$). *B*: trace of holding current shows that H^+ decreases outward current. At pH 7.3, Ba^{2+} decreases holding current and blocks effects of pH on holding current. *C*: trace of holding current from a pH-sensitive astrocyte shows desipramine decreases holding current and blocks effects of pH on holding current. *D*: average ($n = 4$) *I–V* relationship of the pH-sensitive current under control conditions and in the presence of 100 μM Ba^{2+} shows negligible pH-sensitive current in Ba^{2+}. *E*: average ($n = 4$) *I–V* relationship of the pH-sensitive current under control conditions and in the presence of desipramine shows negligible pH-sensitive current in desipramine. *F*: average pH-sensitive current amplitude (at −80 mV) recorded from RTN astrocytes during acidification from pH 7.5 to 6.9, exposure to Ba^{2+} ($n = 3$), or desipramine ($n = 4$) (*$P < 0.05$).


A1
Membrane potential (mV)
-60
-85
H+
des
Ba2+
bath pH
6.9
7.3
A2
Δ Vm (mV)
14
7
0
H+ Ba2+ des
B
I holding current (pA)
150
0
Ba2+
5 min
7.5
6.9
7.3
bath pH
C
I holding current (pA)
100
25
desipramine
5 min
7.5
6.9
7.3
bath pH
D
IpH
300
-300
-150 mV
0 pA
-90
-30
control
Ba2+
E
IpH
300
-300
-150 mV
0 pA
-90
-30
control
desipramine
F
IpH amplitude at -80 mV (pA)
80
40
0
control
Ba2+
Des

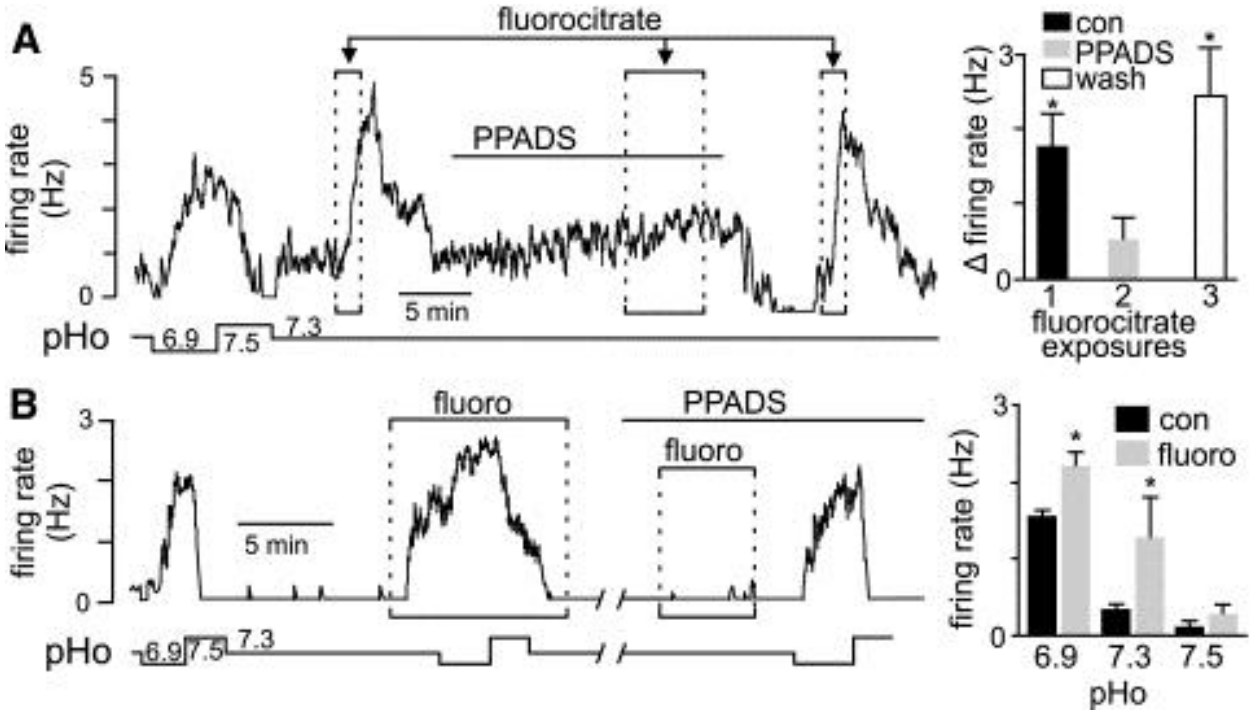
pH-sensitive Kir channels (Su, Ohno et al. 2007, Tang, Taniguchi et al. 2009). Our results indicate that desipramine mimics effects of acidification; in current-clamp exposure to desipramine depolarized membrane potential by 8.4 ± 3.0 mV (n = 4) (Fig. 4*A*) and in voltage-clamp it decreased holding current by 35.0 ± 13 pA (n = 4) (Fig. 4*C*) and the pH-sensitive current by 72.7 ± 21.3% (n = 4) (Fig. 4, *C*, *E*, and *F*). It should be noted that desipramine is also a tricyclic antidepressant and may have nonspecific effects on neuronal activity. However, RTN astrocytes are not responsive to norepinephrine or serotonin (DK Mulkey, unpublished observations), suggesting the effects of desipramine on these cells is mediated by inhibition of Kir4.1-containing channels. These results suggest that heteromeric Kir4.1–Kir5.1 channels confer pH sensitivity to RTN astrocytes.

RTN astrocytes activate pH-sensitive neurons by a purinergic mechanism

Recent evidence suggests that a subset of RTN astrocytes contribute to the mechanism of chemoreception by sensing CO_2/H^+ and releasing ATP to activate pH-sensitive neurons and increase respiratory drive (Gourine, Kasymov et al. 2010). These results are supported by our evidence that roughly 20% of RTN astrocytes are intrinsically pH sensitive. To test the possibility that pH-sensitive astrocytes modulate activity of pH-sensitive neurons, we paralleled previous in vivo experiments by using fluorocitrate to selectively depolarize astrocytes (Erlichman, Li et al. 1998, Holleran, Babbie et al. 2001) while recording activity of pH-sensitive neurons. Fluorocitrate is a metabolic toxin that is selectively taken up by astrocytes, where it can initiate membrane depolarization (Erlichman, Leiter 2010), presumably by energy depletion and loss of cytoplasmic K^+. At subtoxic doses, fluorocitrate has been shown to reversibly depolarize RTN glia and increase ventilatory sensitivity to CO_2 (Erlichman, Li et al. 1998, Holleran, Babbie et al. 2001), by yet unknown mechanisms. We confirmed that fluorocitrate (50 μM) can reversibly depolarize pH-sensitive astrocytes (data not shown). We also confirmed the effects of fluorocitrate are not due to residual Ba^{2+} by determining that Ba^{2+} precipitation control solution, prepared in a manner similar to fluorocitrate, had no effect on neuronal or glial membrane potential. We found that fluorocitrate evoked a large and reversible neuronal activation (Fig. 5, *A* and *B*). Importantly, a second exposure to

Figure 5

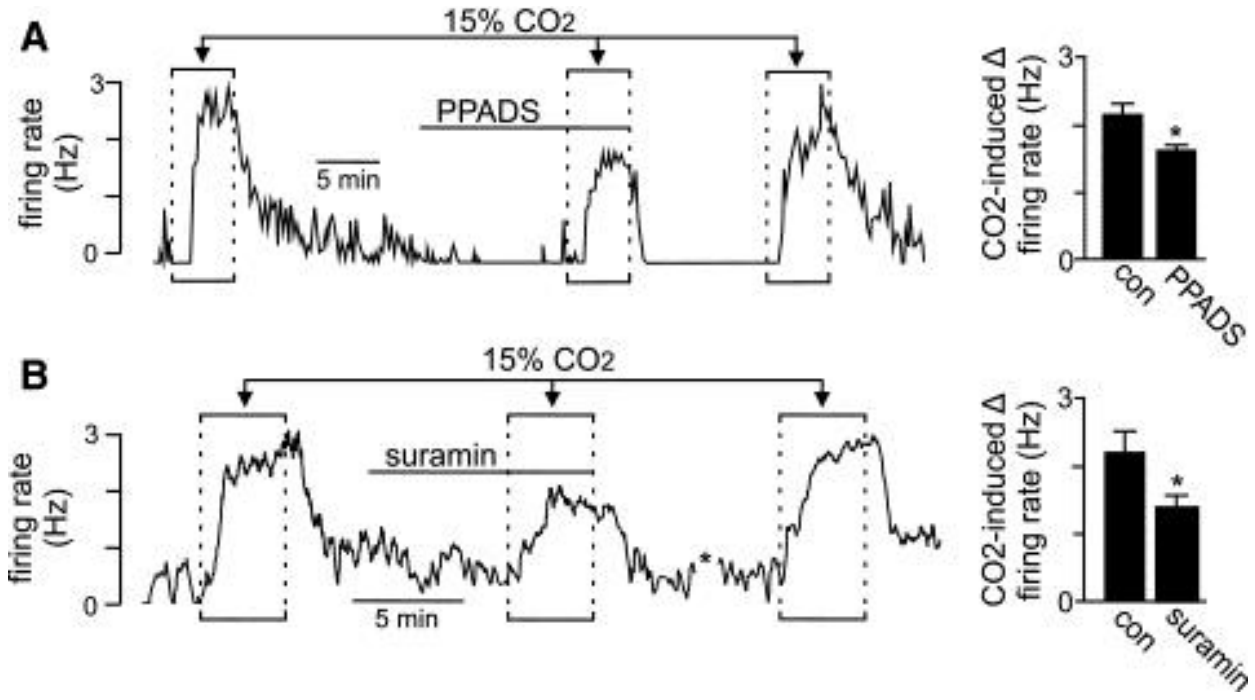
RTN astrocytes increase baseline activity and pH sensitivity of RTN chemoreceptors by a P2-receptor–dependent mechanism. *A*: traces of pH and firing rate (Hz) from a pH-sensitive neuron shows a characteristic response to H^+: increasing pH to 7.5 inhibited activity, whereas acidification to pH 6.9 increased firing rate >2 Hz. At a control pH of 7.3, exposure to fluorocitrate (fluoro, 50 μM) evoked a large and reversible increase in neuronal activity. A second exposure to fluorocitrate, this time in the presence of a P2-receptor antagonist pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate (PPADS), 100 μM, blocked the effects of fluorocitrate. Fluorocitrate responsiveness returned in wash. *B*: traces of pH and firing rate from a pH-sensitive RTN neuron exposed to acidic and alkaline conditions in the presence and absence of fluorocitrate (fluoro, 50 μM) or PPADS (100 μM). Fluorocitrate alone stimulated neuronal activity. In the continued presence of fluorocitrate, acidification further increased firing rate by about 1 Hz, whereas alkalization decreased activity. Note that the effects of pH but not fluorocitrate were retained in the presence of PPADS. Bar graphs summarize the effects of fluorocitrate in the presence of PPADS (*top*, $n = 5$) and in acidic and alkaline conditions (*bottom*, $n = 3$) (*$P < 0.05$).


A
fluorocitrate
PPADS
5 min
firing rate (Hz)
5
0
pHo
6.9
7.5
7.3
con
PPADS
wash
Δ firing rate (Hz)
3
0
1
2
3
fluorocitrate exposures
B
fluoro
PPADS
fluoro
5 min
firing rate (Hz)
3
0
pHo
6.9
7.5
7.3
con
fluoro
firing rate (Hz)
3
0
6.9
7.3
7.5
pHo

fluorocitrate, this time in the presence of an ATP receptor antagonist (PPADS, 100 μM), had no effect on neuronal activity (Fig. 5*A*). In addition, fluorocitrate also increased neuronal pH sensitivity; in the continued presence of fluorocitrate, responsiveness to acidification increased by 0.7 ± 0.2 Hz (n = 3), compared with acid alone (Fig. 5*B*). These results also confirm previous results that P2 receptors are not required for pH sensitivity of RTN neurons in slices incubated in HEPES buffer (Mulkey, Mistry et al. 2006); pH sensitivity of RTN neurons was wholly retained in the presence of PPADS (Fig. 5*B*). It has been suggested that CO_2 and not H^+ per se is the stimulus required for ATP release from RTN astrocytes (Spyer 2009). Therefore we tested the effect of PPADS and another P2-receptor antagonist (suramin, 100 μM) on CO_2/H^+ sensitivity in bicarbonate-buffered medium. As shown in Fig. 6, exposure to 15% CO_2 alone increased neuronal activity 2.2 ± 0.1 Hz (n = 12). After washing to 5% CO_2 exposure to PPADS (100 μM) significantly decreased baseline activity from 0.13 ± 0.05 to 0.02 ± 0.01 Hz (n = 8). Likewise, suramin (100 μM) decreased baseline activity 0.50 ± 0.1 Hz to 0.3 ± 0.1 Hz (n = 4). These results suggest that in bicarbonate buffer (pH 7.3) RTN chemoreceptors receive tonic excitatory purinergic input. Prior to testing CO_2/H^+ sensitivity in PPADS or suramin, a depolarizing current (~1 nA) was delivered to the neuron to approximate the control level of activity. In the continued presence of PPADS (with baseline activity adjusted by DC current injection to near control levels), a second exposure to 15% CO_2 increased neuronal activity by 1.6 ± 0.1 Hz (n = 8); this response was significantly less than the CO_2 response under control conditions (initial response or in wash) (Fig. 6*A*). In the presence of suramin (with baseline activity adjusted to near control levels), a second exposure to 15% CO_2 increased neuronal activity by 1.4 ± 0.1 Hz; this response was significantly less than the CO_2 response under control conditions (Fig. 6*B*). These results indicate that P2-receptor antagonists PPADS and suramin decreased CO_2/H^+ sensitivity. To the extent that fluorocitrate selectively affects astrocytes (Erlichman, Leiter 2010), these results suggest that astrocytes can release ATP and activate pH-sensitive neurons, presumably by a P_2Y-dependent mechanism (Mulkey, Mistry et al. 2006), to increase chemoreceptor output. These results also lend support to the hypothesis that pH-sensitive astrocytes contribute to chemoreception by increasing activity of pH-sensitive neurons.

Figure 6

In bicarbonate-buffered solution astrocytes contribute to chemosensitivity by a purinergic mechanism. *A*: trace of firing rate shows the response of an RTN neuron to increases in CO_2 from 5 to 15% in bicarbonate buffer alone or in the presence of PPADS. An initial exposure to 15% CO_2 increased the firing rate about 2.8 Hz. After returning to 5% CO_2, exposure to PPADS (100 μM) decreased baseline firing rate, suggesting that in bicarbonate buffer RTN chemoreceptors receive tonic excitatory purinergic input. A depolarizing current (~1 nA) was delivered to the neuron to increase firing rate to near control levels. In the continued presence of PPADS (with baseline activity adjusted by DC current injection to near control levels), a second exposure to 15% CO_2 increased the firing rate about 1.5 Hz. After washing, PPADS CO_2 sensitivity returned to initial levels. Bar graph on the *right* summarizes the firing rate response of RTN neurons (n = 9) to 15% CO_2 in control and PPADS (*P < 0.05). *B*: firing rate trace shows the response of a RTN chemoreceptor to 15% CO_2 in bicarbonate-buffered solution alone and in the presence of suramin (100 μM). An initial exposure to 15% CO_2 increased the firing rate about 2.7 Hz. As was observed with PPADS, exposure to suramin (100 μM) in 5% CO_2 decreased baseline firing rate. A depolarizing current (~1 nA) was delivered to the neuron to increase firing rate to near control levels. In the continued presence of suramin (with baseline activity adjusted by DC current injection to near control levels), a second exposure to 15% CO_2 increased firing rate about 1.7 Hz. After washing, suramin CO_2 sensitivity returned to initial levels. The asterisk designates a 5-min break. Bar graph on the *right* summarizes the firing rate response of RTN neurons (n = 5) to 15% CO_2 in control and suramin (*P < 0.05).


A
15% CO2
PPADS
5 min
firing rate (Hz)
3
0
CO2-induced Δ firing rate (Hz)
con
PPADS
*
B
15% CO2
suramin
5 min
firing rate (Hz)
3
0
CO2-induced Δ firing rate (Hz)
con
suramin
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DISCUSSION

Neuron–glia communication has emerged in recent years as a fundamental process in the brain that can be an important determinant of neuronal activity. However, by most accounts the activity of astrocytes is thought to be under the strict control of neuronal input (Fellin, Pascual et al. 2006). Here we show that RTN astrocytes sense H^+ in part by inhibition of heteromeric Kir4.1–Kir5.1 channels and stimulate activity of RTN chemoreceptors by a P2-receptor–dependent mechanism. Our evidence also indicates that purinergic input is not required for RTN chemoreceptor function. These results identify the most likely mechanism by which RTN astrocytes sense pH and build on recent evidence that astrocytes contribute to respiratory drive by a purinergic mechanism (Gourine, Kasymov et al. 2010).

Molecular mechanism underlying pH sensing by RTN astrocytes

Fukuda and colleagues showed >30 years ago that the RTN contains a population of pH-sensitive nonspiking cells (Fukuda, Honda et al. 1978) and more recent evidence shows these pH sensors express the NBC (Ritucci, Erlichman et al. 2005), thus narrowing the scope of candidates to either astrocytes or oligodendrocytes. Our data indicate that pH-sensitive RTN glia are protoplasmic astrocytes; specifically, these cells have a hyperpolarized membrane potential; high K^+ conductance; exhibit an electrically passive profile; and express Aldh1L1, a marker of protoplasmic astrocytes (Cahoy, Emery et al. 2008).

Several pH-sensitive channels have been proposed to contribute to the mechanism of chemoreception, including Ca^{+2}-activated K^+ channels (K_{Ca}) (Wellner-Kienitz, Shams et al. 1998), voltage-sensitive K^+ channels (e.g., transient and delayed rectifying K^+ channels)(Putnam, Filosa et al. 2004, Filosa, Putnam 2003), background TASK channels (Bayliss, Talley et al. 2001, Mulkey, Stornetta et al. 2004), and Kir channels(Schultz, Czachurski et al. 2003, Zhu, Liu et al. 2000, Xu, Cui et al. 2000). All of these channels are expressed in various types of glial cells and conceivably contribute to the pH sensitivity of RTN astrocytes. Our results show that the pH-sensitive current in RTN astrocytes can be blocked with 100 μM Ba^{2+}, which will fully block most Kir channels, with only modest effects on background K^+ channels

(e.g., TASK channels) (Goldstein, Bayliss et al. 2005, Kubo, Adelman et al. 2005, Patel, Honore 2001). In addition, the pH-sensitive current in RTN astrocytes was not blocked by TEA (10 mM) or 4-AP (50 μM), which can inhibit other voltage- and Ca^{2+}-dependent K^+ channels, thus narrowing the scope of candidate molecular sensors to one or more Kir channels.

The Kir family of channels consists of 16 different channel subunits grouped into seven subfamilies (Kir1.x–Kir7.x) based on sequence homology (Olsen, Sontheimer 2008). All Kir channels are composed of four α-subunits assembled as homomeric or heteromeric channels that show varying degrees of rectification, single-channel conductance, and pharmacology. A common feature of most Kir channels is high sensitivity to low micromolar concentrations of Ba^{2+} (Kubo, Adelman et al. 2005, Mulkey, Stornetta et al. 2004), a property that is frequently used to differentiate contributions of Kir channels from other K^+ channels, including background K^+ channels (Mulkey, Stornetta et al. 2004). Only certain Kir channels are pH sensitive, e.g., Kir1.1 (pK = 6.7) (Xu, Yang et al. 2000), Kir2.3 (pK = 6.8) (Putnam, Filosa et al. 2004), homomeric Kir4.1 (pK = 6.1) (Pessia, Imbrici et al. 2001), and the heterodimeric Kir4.1–Kir5.1 channels (pK = 7.4) (Casamassima, D'Adamo et al. 2003, Xu, Yang et al. 2000). Note that Kir5.1 subunits do not form functional homomeric channels, but preferentially heteromerize with Kir4.1 subunits in astrocytes (Casamassima, D'Adamo et al. 2003, Pessia, Tucker et al. 1996). Of all pH-sensitive Kir channels, only heteromeric Kir4.1–Kir5.1 channels are exquisitely sensitive to physiologically relevant pH changes. Further, in situ hybridization found that the RTN expresses high levels of Kir4.1 and Kir5.1 mRNA (Wu, Xu et al. 2004). Here, we show that RTN astrocytes respond to physiologically relevant pH changes. The pH-sensitive current expressed by these cells appears relatively voltage independent between −150 and −30 mV and was almost completely blocked by micromolar concentrations of Ba^{2+} and desipramine (a blocker of Kir4.1-containing channels; (Su, Ohno et al. 2007, Tang, Taniguchi et al. 2009)). This combination of features (i.e., inhibition by physiologically relevant changes in pH and micromolar concentrations of Ba^{2+} and desipramine) is most consistent with heteromeric Kir4.1–Kir5.1

channels. Therefore we consider heteromeric Kir4.1–Kir5.1 channels to be excellent candidate pH sensors of RTN astrocytes.

Our results also indicate that the NBC contributes to the pH-sensitive current in RTN astrocytes. It is well established in various types of astrocytes that activity of the NBC generates a hyperpolarizing outward current (i.e., net HCO_3^- influx) at membrane potentials depolarized to E_{NBC} (Chesler 2003). In our experimental conditions we calculated the E_{NBC} to be about −99 mV. As expected, at a holding potential of −80 mV in bicarbonate-buffered solution we observed a DIDS-sensitive outward current. This outward current will likely increase during depolarization and so potentially blunt H^+-mediated inhibition of Kir4.1–Kir5.1 and the corresponding membrane potential. However, the NBC is also modulated by changes in extracellular pH; bath acidification can reverse Na^+-HCO_3^- transport to produce an inward depolarizing current (i.e., net HCO_3^- efflux) (Chesler 2003, Deitmer 1991). In voltage-clamp (at a holding potential of −80 mV), we show that inward current produced by the NBC increased amplitude of the CO_2/H^+-sensitive current and caused a negative shift in its reversal potential. When activity of the NBC was blocked with DIDS, we observed a CO_2/H^+-sensitive current that was similar in amplitude to that of the pH-sensitive current in HCO_3^--free HEPES solution and reversed near E_{K^+}. A contribution of the NBC to central chemoreception has yet to be established and considering that H^+-induced depolarization and extracellular acidification have offsetting effects on directionality of Na^+/HCO_3^- transport by the NBC, further experiments are required to define potential roles of this transporter in RTN chemoreception.

Possible role of astrocytes in central chemoreception

There are two mechanisms by which astrocytes are thought to influence chemoreception. First, a well-known function of astrocytes is to help regulate extracellular K^+ by taking up extracellular K^+ ions during increased neuronal activity (Olsen, Sontheimer 2008). This process effectively buffers changes in extracellular K^+; however, it can also depolarize membrane potential and increase Na^+/HCO_3^- cotransport

into glia (Chesler 2003), which would enhance the CO_2-induced fall in extracellular pH and potentiate activation of chemoreceptors. This possibility is supported by a series of in vivo experiments in which fluorocitrate microinjected into the RTN caused extracellular acidification followed by increased respiratory frequency and ventilatory sensitivity to CO_2 (Erlichman, Leiter 2010, Holleran, Babbie et al. 2001). Note that fluorocitrate is a toxin that inhibits metabolic activity; at low concentrations it has been shown to be selectively taken up by astrocytes but not neurons, where it initiates membrane depolarization (Erlichman, Leiter 2010), presumably by loss of cytoplasmic K^+. However, it is also possible that the fluorocitrate-mediated effects described in these experiments resulted from the release of excitatory gliotransmitters acting on pH-sensitive neurons. Our data support this latter possibility. For example, we found that exposure to fluorocitrate activated pH-sensitive neurons under conditions in which the NBC is minimally active (i.e., in HEPES buffer). In addition, we were able to block effects of fluorocitrate with a P2-receptor antagonist. Therefore the most likely mechanism by which astrocytes contribute to chemoreception involves release of ATP and activation of pH-sensitive neurons. Our data also indicate that fluorocitrate caused an upward parallel shift in the pH sensitivity of RTN neurons, suggesting that fluorocitrate-mediated ATP release from RTN astrocytes contributes to chemoreception by increasing excitability of RTN neurons. These results are consistent with evidence in other brain regions that astrocytes serve as a source of excitatory purinergic drive able to rapidly modulate neural activity (Gordon, Iremonger et al. 2009). However, it should be noted that these experiments are limited by a dearth of pharmacological tools available to selectively manipulate astrocytes and the possibility that fluorocitrate may have nonspecific effects on other cell types, including neurons. Therefore additional experiments that use genetic approaches to selectively manipulate astrocytes are required to definitively establish a role of pH-sensitive astrocytes in chemoreception. It is important to note that our results are entirely consistent with a recent study that used the adenoviral system to target expression of ChR2 to RTN astrocytes (Gourine, Kasymov et al. 2010). This study showed that photostimulation of RTN astrocytes increased activity of RTN neurons and stimulated breathing by a purinergic-dependent

mechanism. These results are similar to the observed effects of fluorocitrate, thus adding to our confidence that fluorocitrate effects are mediated by astrocytes.

Based on the results described earlier, it is reasonable to expect P2-receptor antagonists to decrease pH sensitivity of RTN neurons. However, previous experiments performed in HEPES-buffered media (Mulkey, Mistry et al. 2006) indicate that PPADS had no effect on pH sensitivity of RTN neurons. A possible explanation for this apparent discrepancy is that CO_2, not H^+, is the effector responsible for hypercapnic-induced ATP release (Spyer, Gourine 2009). Therefore in a parallel series of experiments, we tested the effects of two P2-receptor antagonists (PPADS and suramin) on CO_2/H^+ sensitivity of RTN neurons in bicarbonate-buffered media. Interestingly, we found that under these conditions both P2-receptor antagonists blunted neuronal CO_2/H^+ sensitivity. These results are consistent with the possibility that CO_2 is the required stimulus for ATP release in the RTN.

In summary, we demonstrate that pH-sensitive RTN glia are protoplasmic astrocytes that sense H^+ by inhibition of heteromeric Kir4.1–Kir5.1-like conductance and contribute to respiratory drive by increasing activity of pH-sensitive neurons by a purinergic-dependent mechanism. Our results also suggest that the NBC may contribute to the pH-sensitive current in RTN astrocytes. Thus we conclude that RTN astrocytes contribute to the mechanism of chemoreception and influence integrated output of the RTN.

Chapter 5 Regulation of Ventral Surface CO2/H+-sensitive neurons by purinergic signaling

INTRODUCTION

Carbon dioxide provides the primary stimulus to breathe. Central respiratory chemoreceptors sense changes in tissue CO_2, H^+ and/or HCO_3^- to regulate the rate and depth of breathing (for review see (Huckstepp, Dale 2011)). It is now well established that the retrotrapezoid nucleus (RTN) is an important site of chemoreception. Specifically, the RTN contains a subset of neurons that is highly CO_2/H^+ sensitive *in vivo* and *in vitro*, is glutamatergic and projects to respiratory centres to directly influence breathing (Mulkey, Stornetta et al. 2004, Takakura, Moreira et al. 2008, Ritucci, Erlichman et al. 2005, Nattie, Fung et al. 1993, Abbott, Stornetta et al. 2009, Weston, Stornetta et al. 2004). The mechanism by which RTN neurons sense pH involves inhibition of an unidentified voltage-independent K^+ conductance (Mulkey, Stornetta et al. 2004, Mulkey, Talley et al. 2007). Evidence also indicates that purinergic signalling contributes to the ventilatory response to CO_2 (Gourine, Llaudet et al. 2005, Gourine, Atkinson et al. 2003, Thomas, Spyer 2000, Thomas, Ralevic et al. 1999). At the level of the RTN, hypercapnia has been shown to evoke the discrete release of ATP near the ventral medullary surface (Huckstepp, id Bihi et al. 2010, Gourine, Kasymov et al. 2010, Gourine, Llaudet et al. 2005), and ATP has been shown to contribute to respiratory drive by increasing activity of CO_2/H^+-sensitive RTN neurons (Mulkey, Mistry et al. 2006, Gourine, Kasymov et al. 2010, Wenker, Kreneisz et al. 2010).

There is increasing evidence that astrocytes are the source of purinergic drive to RTN chemoreceptors (Huckstepp, id Bihi et al. 2010, Wenker, Kreneisz et al. 2010, Gourine, Kasymov et al. 2010); however, the extent to which ATP contributes to RTN chemoreception is questionable and the mechanisms underlying purinergic modulation of RTN chemoreceptors are not clear (Mulkey, Wenker 2011). For example, a recent study reported that H^+ sensitivity of RTN neurons could be blocked with a P2 receptor antagonist (Gourine, Kasymov et al. 2010), suggesting that RTN chemoreception is entirely dependent on purinergic signalling. The same study also suggested that the purinergic drive to RTN neurons is

dependent on extracellular Ca^{2+}; it showed that acidification triggered a Ca^{2+} wave that propagated between ventral surface astrocytes, possibly by activating Ca^{2+}-permeable channels (e.g. P2X1 or P2X3), to potentiate additional ATP release and subsequently activate Phox2b-expressing RTN neurons *in vitro* and increased respiratory activity *in vivo*. Conversely, we have shown *in vitro* that RTN neurons are highly pH sensitive in the presence of P2 receptor blockers, and that purinergic signalling contributes to only a portion of neuronal CO_2/H^+ sensitivity (Mulkey, Mistry et al. 2006, Wenker, Kreneisz et al. 2010). Still further evidence obtained using the brainstem–spinal cord preparation reported that purinergic signalling does not contribute to CO_2 responsiveness of neurons in the parafacial respiratory group (pFRG)/RTN of newborn rats (Onimaru, Ikeda et al. 2012). In addition, the mechanism underlying CO_2-evoked ATP release in the RTN appears to be mediated by direct gating of gap junction hemichannels in a Ca^{2+}-independent manner (Huckstepp, id Bihi et al. 2010).

The objectives of this study are to establish the extent to which purinergic signalling contributes to RTN chemoreception both *in vivo* and *in vitro*, and to gain insight into the mechanisms underlying CO_2-evoked ATP release and downstream activation of RTN neurons. To make these determinations, we recorded phrenic nerve activity *in vivo* or the firing rate response of CO_2/H^+-sensitive RTN neurons in the brain slice preparation during exposure to hypercapnia alone and in the presence of P2 receptor blockers. In addition, we used a pharmacological approach to manipulate potential mechanisms of ATP release and activity of downstream P2 receptors. We find that purinergic signalling is an important component of RTN chemoreception; application of P2 receptor antagonists into the RTN reduced the ventilatory response to CO_2 by ~30% *in vivo*, and blunted the firing rate response of RTN neurons to both 10% and 15% CO_2 also by 30%. This purinergic drive to breathe was independent of temperature, stimulus strength and changes in the extracellular Ca^{2+} gradient, but could be blocked with gap junction hemichannel antagonists. These results are most consistent with a recent study by Huckstepp and colleagues (Huckstepp, id Bihi et al. 2010), and suggest that astrocytes but not neurons mediate CO_2-evoked ATP release by a mechanism involving gap junction hemichannels.

METHODS

In vivo preparation

Animal use was in accordance with guidelines approved by the University of São Paulo Animal Care and Use Committee and conforms to the principles of UK regulations, as described in (Drummond 2009). All efforts were made to minimize the number of animals used and their suffering. All *in vivo* experiments were performed in male Wistar rats weighing 250–280 g; a total of 38 rats were used for these *in vivo* experiments. The surgical procedures and experimental protocols were similar to those previously described (Takakura, Moreira et al. 2011, Takakura, Moreira 2011). Briefly, general anaesthesia was induced with 5% halothane in 100% O_2. A tracheostomy was made and the halothane concentration was reduced to 1.4–1.5% until the end of surgery. The femoral artery was cannulated (polyethylene tubing, 0.6 mm o.d., 0.3 mm i.d., Scientific Commodities, Lake Havasu City, AZ, USA) for measurement of arterial pressure (AP). The femoral vein was cannulated for administration of fluids and drugs. The occipital plate was removed, and a micropipette was placed in the medulla oblongata via a dorsal transcerebellar approach for microinjection of drugs. A skin incision was made over the lower jaw for placement of a bipolar stimulating electrode, next to the mandibular branch of the facial nerve, as previously described (Moreira, Takakura et al. 2006, Takakura, Moreira et al. 2011). The phrenic nerve was accessed by a dorsolateral approach after retraction of the right shoulder blade. To prevent any influence of artificial ventilation on phrenic nerve activity (PNA), the vagus nerve was cut bilaterally. Additionally, a complete baro- and peripheral chemoreceptor deafferentation was performed by sectioning the vagosympathetic trunks, the superior laryngeal nerves and the glossopharyngeal nerves (proximal to the junction with the carotid sinus nerves). Upon completion of the surgical procedures, halothane was replaced with urethane (1.2 g kg^{-1}) administered slowly i.v. All rats were ventilated with 100% O_2 throughout the experiment. Rectal temperature was maintained at 37°C. End-tidal CO_2 was monitored throughout each experiment with a capnometer (CWE, Inc., Ardmore, PA, USA) that was calibrated twice per experiment with a calibrated CO_2–N_2 mix. This instrument provided a reading of <0.1% CO_2 during inspiration in animals

breathing 100% O_2, and an asymptotic, nearly horizontal reading during expiration. The adequacy of anaesthesia was monitored during a 20 min stabilization period by testing for the absence of withdrawal responses, pressor responses and changes in PNA to a firm toe pinch. After these criteria were satisfied, the muscle relaxant pancuronium was administered at an initial dose of 1 mg kg^{-1} i.v. and the adequacy of the anaesthesia was thereafter gauged solely by the lack of increase in AP and PNA rate or amplitude to a firm toe pinch. Approximately hourly supplements of one-third of the initial dose of urethane were needed to satisfy these criteria throughout the recording period (2 h).

***In vivo* recordings of physiological variables**

As previously described (Mulkey, Stornetta et al. 2004, Moreira, Takakura et al. 2006, Takakura, Moreira et al. 2011), mean arterial pressure (MAP), phrenic nerve activity (PNA) and end-expiratory CO_2 ($etCO_2$) were digitized with a micro1401 (Cambridge Electronic Design), stored on a computer and processed off-line with version 6 of Spike 2 software (Cambridge Electronic Design). Integrated phrenic nerve activity (iPNA) was obtained after rectification and smoothing (τ = 0.015 s) of the original signal, which was acquired with a 30–300 Hz bandpass filter. PNA amplitude (PNA amp) and PNA frequency (PNA freq) were expressed for each rat on a scale from 0 (value during apnoea) to 100 (value while breathing 10% CO_2).

Hypercapnia was produced by addition of pure CO_2 to the 100% O_2 supplied by artificial ventilation to increase the maximum end-expiratory CO_2 to 9.5–10%. End-expiratory CO_2 was maintained for 5 min, and CO_2 was removed. Each rat was submitted to three sessions of hypercapnia: one session 10 min after bilateral saline injections into the RTN, and two additional sessions, 10 and 60 min after bilateral injections of saline, PPDAS or MRS2179 into the RTN.

Histology

At the end of each *in vivo* experiment, rats were deeply anaesthetized with halothane and perfused through the heart with PBS (pH 7.4) followed by paraformaldehyde (4% in 0.1 m phosphate buffer, pH

7.4). The brains were removed and stored in fixative for 24 h at 4°C. The medulla was cut in 40-μm-thick coronal sections with a vibrating microtome (Vibratome 1000S Plus, USA). Sections were stored at −20°C in a cryoprotectant solution. The injection sites were confirmed with an Axioskop 2 microscope (Zeiss, Oberkochen, Germany). Sections from different brains were aligned with respect to a reference section, which was the most caudal section containing an identifiable cluster of facial motor neurons. To this reference section was assigned a value of 11.6 mm caudal to bregma (bregma −11.6 mm, (Paxinos, Watson 1982)). Levels rostral or caudal to this reference section were determined by adding or subtracting the number of intervening sections × 40 μm.

Brain slice preparation

All procedures were performed in accordance with National Institutes of Health and University of Connecticut Animal Care and Use Guidelines and conformed to the principles of UK regulations, as described in (Drummond 2009). A total of 64 rat pups were used for these *in vitro* experiments. Slices containing the RTN were prepared as previously described (Mulkey, Mistry et al. 2006, Wenker, Kreneisz et al. 2010). Briefly, neonatal rats (7–12 days postnatal) were decapitated under ketamine–xylazine anaesthesia and transverse brainstem slices (300 μm) were cut using a microslicer (DSK 1500E; Dosaka, Kyoto, Japan) in ice-cold substituted Ringer solution containing (in mm): 260 sucrose, 3 KCl, 5 $MgCl_2$, 1 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose and 1 kynurenic acid. Slices were incubated for ∼30 min at 37°C and subsequently at room temperature in normal Ringer solution (in mm): 130 NaCl, 3 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$ and 10 glucose. Both substituted and normal Ringer solutions were bubbled with 95% O_2–5% CO_2 (extracellular pH (pH_o) 7.35).

Slice-patch electrophysiology

Individual slices were transferred to a recording chamber mounted on a fixed-stage microscope (Zeiss Axioskop FS) and perfused continuously (∼2 ml min^{-1}) with a bath solution of normal Ringer solution (same as incubation Ringer solution above) bubbled with 95% O_2–5% CO_2 (pH_o 7.35). The pH of the

bicarbonate-based bath solution was decreased to 6.90 by bubbling with 15% CO_2 or 7.10 by bubbling with 10% CO_2. All recordings were made with an Axopatch 200B patch-clamp amplifier, digitized with a Digidata 1322A A/D converter, and recorded using pCLAMP 10.0 software (Molecular Devices). Recordings were obtained at room temperature (~22°C) with patch electrodes pulled from borosilicate glass capillaries (Warner Instruments) on a two-stage puller (P89; Sutter Instrument) to a DC resistance of 4–6 MΩ when filled with an internal solution containing the following (in mm): 120 KCH_3SO_3, 4 NaCl, 1 $MgCl_2$, 0.5 $CaCl_2$, 10 Hepes, 10 EGTA, 3 Mg-ATP and 0.3 GTP-Tris (pH 7.2) plus 0.2% biocytin; electrode tips were coated with Sylgard 184 (Dow Corning). All recordings of neuronal firing rate were performed in the loose-patch configuration to ensure minimal alteration of the intracellular milieu. Firing rate histograms were generated by integrating action potential discharge in 10 s bins and plotted using Spike 5.0 software. Excitatory postsynaptic currents (EPSCs) were measured from chemosensitive neurons (identified in loose-patch) in whole-cell voltage clamp (I_{hold} = −60 mV) and analysed off-line with AxoGraph X using the following parameters: amplitude = −25 pA, rise constant = 0.7 ms and decay constant = 1.6 ms, with a threshold of detection set to −7 SD.

Drugs

All drugs were purchased from Sigma. For *in vivo* experiments, the non-specific P2 receptor antagonist pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), the P2Y1-receptor antagonist MRS2179 and the P2Y1-receptor agonist MRS2365 were diluted to 100 μm in sterile saline (pH 7.4) and injected into the RTN using single-barrel glass pipettes (tip diameter of 20 μm) connected to a pressure injector (Picospritzer III, Parker Hannifin Corp, Cleveland, OH, USA). For each injection we delivered a volume of 50 nl over a period of 5 s. These glass pipettes also allowed recordings of field potential properties that were used to help direct the electrode tip to the desired site. Injections in the RTN region were guided by recordings of the facial field potential (Brown, Guyenet 1985), and were placed 250 μm below the lower edge of the field, 1.7 mm lateral to the midline and 200 μm rostral to the caudal end of the field. Recordings were made on one side only; the second injection was made 1–2 min later at the same level on

the contralateral side. We included a 5% dilution of fluorescent latex microbeads (Lumafluor, New City, NY, USA) with all drug applications to mark the injection sites and verify the spread of the injections (Takakura, Moreira et al. 2011, Takakura, Moreira 2011). For the *in vitro* experiments, we bath applied PPADS (100 μm) or suramin (100 μm) to block P2 receptors, MRS2179 (3 μm) to block P2Y1 receptors, and carbenoxolone (CBX, 100 μm) or cobalt (500 μm) to block gap junction hemichannels. A low Ca^{2+}, high Mg^{2+} synaptic block solution was used to block potential ATP release from neurons and to limit Ca^{2+} influx into astrocytes from the extracellular space. The composition of the synaptic block medium used in this study is similar to normal Ringer solution except that $MgCl_2$ was increased to 11.4 mm, $CaCl_2$ was decreased to 0.2 mm, and to maintain osmolality, NaCl was decreased to 124 mm. The efficacy of this synaptic blocking medium is well established (Hatton 1982, Richards, Sercombe 1970, Mason 1980) and confirmed here by recording its effects on spontaneous EPSCs. In addition, the specific P2Y1 receptor agonist (MRS2365; 100 μm in Hepes-buffered medium, pH 7.3) was delivered focally using low-resistance pipettes connected to a Picospritzer III (Parker Instrumentation, Cleveland, OH, USA) and manoeuvered into close proximity of the target neurons. Application times were 600 ms, and vehicle control experiments were performed to ensure agonist responses were not attributable to pressure artifacts.

Statistics

Data are reported as mean ± standard error of the mean. Statistical analysis was performed using Sigma Stat version 3.0 software (Jandel Corporation, Point Richmond, CA, USA). *t* test, paired *t* test or one-way ANOVA followed by the Newman–Keul multiple comparisons test were used as appropriate ($P < 0.05$).

RESULTS

This study consists of three sets of experiments. First, to determine the extent to which purinergic signalling contributes to central chemoreception, my collaborators at the University of Sao Paulo tested the effects of P2 receptor antagonists (PPADS or suramin) on the hypercapnic ventilatory response *in*

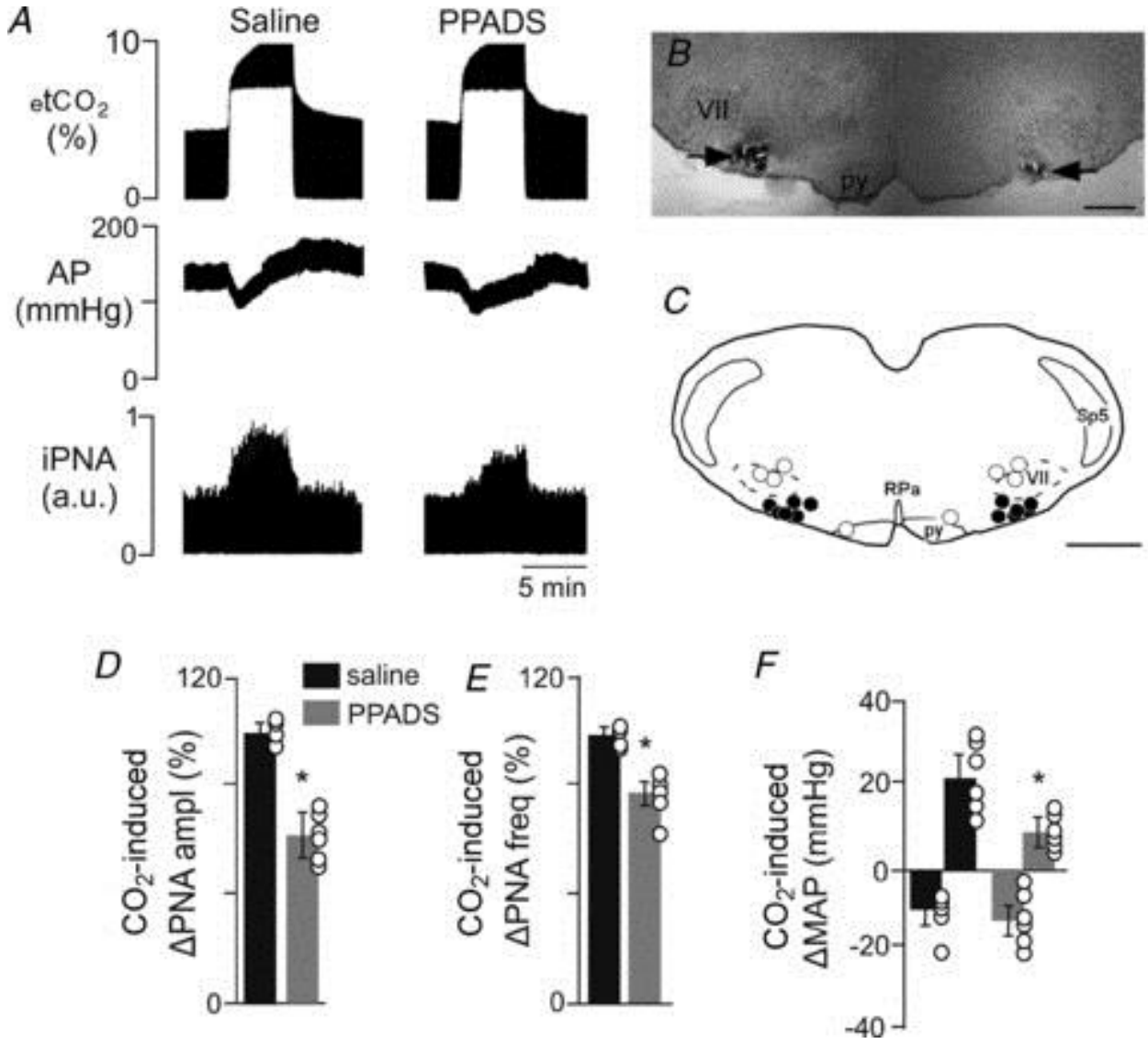
vivo, and on the firing rate response of RTN neurons to 10% and 15% CO_2 at room or body temperature *in vitro*. Second, to determine if purinergic drive to RTN neurons is dependent on synaptic input or requires extracellular Ca^{2+}, as previously reported, I compared effects of P2 receptor antagonists on neuronal CO_2/H^+ responsiveness under control conditions and in low Ca^{2+}–high Mg^{2+} synaptic block medium. Third, to determine if purinergic drive to CO_2/H^+-sensitive RTN neurons is mediated by hemichannels, I tested neuronal CO_2/H^+ sensitivity in the presence of the gap junction blockers CBX or cobalt. Note that chemosensitive RTN neurons have been shown to express the transcription factor Phox2b (Stornetta, Moreira et al. 2006, Lazarenko, Milner et al. 2009); however, Phox2b has also been shown to be expressed by other cells in relatively close proximity to the RTN, including catecholaminergic neurons (C1 and A5), facial motor neurons and the superior salivatory nucleus (Kang, Chang et al. 2007). Therefore, we chose to functionally identify CO_2/H^+-sensitive RTN neurons based on their characteristic firing rate response to CO_2/H^+. Neurons were considered chemosensitive if they were spontaneously active in 5% CO_2 (pH_o 7.35) and responded to 15% CO_2 (pH_o ~6.90) with at least 1.5 Hz increase in firing rate. This level of CO_2/H^+ responsiveness is similar to what we, and others, have reported for RTN chemosensitive neurons (Ritucci, Erlichman et al. 2005, Wenker, Kreneisz et al. 2010, Mulkey, Mistry et al. 2006). RTN neurons that did not exhibit this minimum firing rate response to 15% CO_2 were considered non-chemosensitive and excluded from this study.

Purinergic drive to RTN chemoreceptors *in vivo*

As expected, when urethane-anaesthetized, sino-aortic denervated and vagotomized rats (n = 6 per group) are exposure to hypercapnia (10% CO_2) PNA amplitude and PNA frequency increased by 100 ± 2% and 99 ± 2% (Fig. 7*A*, *D* and *E*). Injections of PPADS (3 mm in 50 nl) were placed bilaterally in the RTN in these rats (Fig. 7*B* and *C*). The injection centre was 250 μm below the facial motor nucleus and 200 μm rostral to the caudal end of this nucleus, targeting the region that contains the highest density of CO_2-sensitive RTN neurons (Mulkey, Stornetta et al. 2004, Takakura, Moreira et al. 2011, Takakura, Moreira 2011). Application of PPADS into the RTN did not change resting PNA activity (99 ± 7% of control

Figure 7

P2 receptor antagonist injections into the RTN attenuate effects of hypercapnia on arterial pressure and PNA in anaesthetized vago-sino-aortic denervated rats. *A*, recordings from one rat showing the effect of injection of PPADS into the RTN on changes in arterial pressure (AP) and phrenic nerve activity (PNA) elicited by an increase of end-expiratory CO_2 from 5 to 10%. Responses were recorded 10 min after bilateral injection of saline into the RTN, and 10 min after bilateral injection of PPADS (3 mm, 50 nl each side) in the RTN. *B*, photomicrograph of a coronal section showing bilateral injections in the RTN. Scale bar is 500 μm. *C*, computer-assisted plot of the centre of injection sites revealed by presence of fluorescent microbeads included in the injectate (coronal plane at Bregma −11.6; (Paxinos, Watson 1982)). Scale bar is 1 mm. *D–F*, summary data ($n = 6$) show changes in PNA amplitude (ΔPNA ampl) (*D*), PNA frequency (ΔPNA freq) (*E*) and mean arterial pressure (ΔMAP) (*F*) elicited by stepping the end-expiratory CO_2 from 5 to 10% during saline or PPADS injections into the RTN. Differences expressed as a percentage of the response to the CO_2 challenge elicited during saline injection. *Different from saline ($P < 0.05$). Abbreviations: py, pyramid; RPa, raphe pallidus; Sp5, spinal trigeminal tract; VII, facial motor nucleus.


A
Saline
PPADS
10
etCO2 (%)
0
200
AP (mmHg)
0
1
iPNA (a.u.)
0
5 min
B
VII
py
C
Sp5
RPa
VII
py
D
120
saline
PPADS
CO2-induced ΔPNA ampl (%)
0
E
120
CO2-induced ΔPNA freq (%)
0
F
40
20
0
-20
-40
CO2-induced ΔMAP (mmHg)

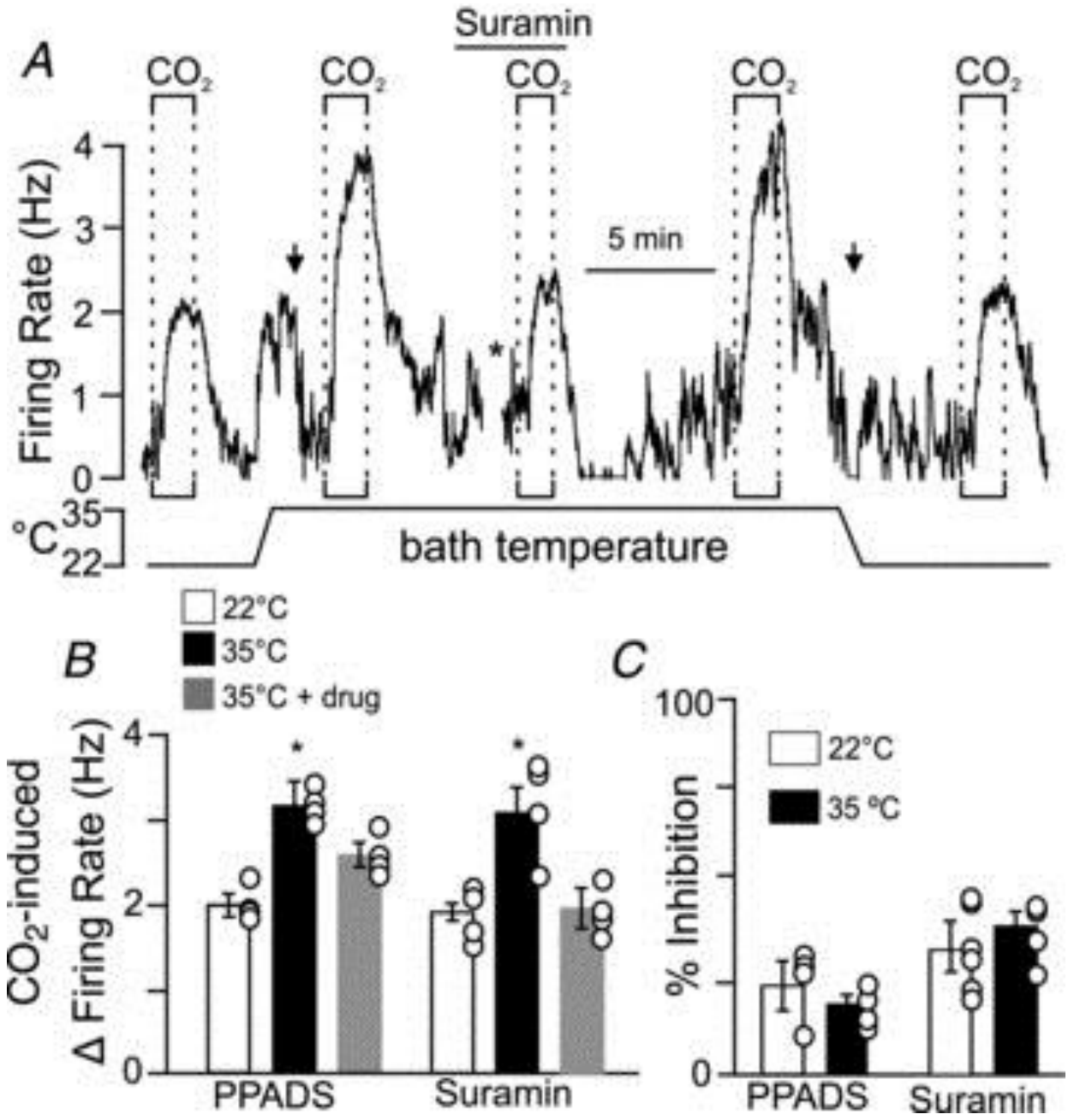
value; $P > 0.05$), but did reduce the hypercapnia-induced increase in PNA amplitude (62 ± 9% of the control) and PNA frequency (77 ± 3% of the control) (Fig. 7*D* and *E*). As shown in Fig. 7*A*, exposure to hypercapnia also caused hypotension (−11 ± 7 mmHg) followed by a gradual return of mean arterial pressure (MAP) to control level, 30 to 40 s later. Immediately after hypercapnia ended, the MAP increased by 21 ± 6 mmHg, and returned to control values within 5 min (Fig. 7*A* and *F*). Injection of PPADS into the RTN did not change resting MAP (124 ± 6 mmHg compared with saline 126 ± 5 mmHg), but did reduce the hypercapnia-induced pressor response from 21 ± 6 to 8 ± 4 mmHg (Fig. 7*A* and *F*). Injections located outside the RTN region often (3 out of 4) reached the facial motor nucleus or dorsal to it and one injection (1 out of 4) was located in the parapyramidal region (data not shown). Bilateral injections of PPADS in the facial motor nucleus or in the parapyramidal region did not change the hypercapnic-induced respiratory or pressor responses in these animals (data not shown).

Purinergic drive to RTN chemoreceptors *in vitro*

Previous evidence indicates that temperature greatly increased pH sensitivity of RTN neurons *in vitro* (Guyenet, Mulkey et al. 2005). Therefore, we wanted to determine if the contribution of purinergic drive to CO_2/H^+-sensitive RTN neurons also increased with temperature. We found that increasing temperature from ~22°C to 35°C increased baseline activity by 2.01 ± 0.18 Hz (Fig. 8*A*) and increased the firing rate response to 15% CO_2 from 1.9 ± 0.09 Hz to 3.3 ± 0.23 Hz ($n = 4$) (Fig. 8*B*). The temperature coefficient (Q_{10}) for the CO_2/H^+ sensitivity of RTN neurons was estimated to be ~1.5 ± 0.1 ($n = 9$); this value is very similar to the pH-sensitive Q_{10} of RTN neurons over a similar pH range (~1.55, extrapolated from Fig. 8*D*; (Guyenet, Mulkey et al. 2005)]. At 35°C, exposure to the P2 receptor antagonists PPADS (100 μm) or suramin (100 μm) decreased firing rate by 0.73 ± 0.18 Hz, consistent with the hypothesis that CO_2/H^+-sensitive RTN neurons receive tonic purinergic input (Wenker, Kreneisz et al. 2010). Prior to testing CO_2/H^+ sensitivity at 35°C, a hyperpolarizing current (~5 pA) was delivered to approximate the control level of neuronal activity. In the continued presence of PPADS or suramin at 35°C (with baseline activity adjusted by DC current injection to near-control levels), exposure to 15% CO_2 increased neuronal activity

Figure 8

Purinergic contribution to CO_2/H^+ sensitivity of RTN neurons is not temperature dependent. *A*, traces of firing rate and bath temperature show CO_2/H^+ responsiveness at room temperature (~22°C) and 35°C with and without suramin (Sur, 100 μm), a P2 receptor antagonist. At room temperature, increasing bath CO_2 from 5 to 15% increased firing rate ~2 Hz. Heating to 35°C increased firing rate ~2 Hz. Prior to testing CO_2/H^+ sensitivity at 35°C we delivered a hyperpolarizing current (−5 nA; left arrow) into the cell to match firing rate to control activity. Under these conditions exposure to 15% CO_2 increased firing rate ~3.5 Hz. A 10 min incubation in suramin (*) decreased the firing rate response to 15% CO_2 by 40%. CO_2/H^+ sensitivity returned to control levels in wash. *B*, average data show the CO_2/H^+-induced firing rate response at room temperature (a portion of these results were published previously Wenker *et al.* 2010) and at 35°C with or without the P2 receptor antagonist PPADS (100 μm, $n = 4$) or suramin (100 μm, $n = 4$). Based on these results we calculated the Q_{10} of CO_2/H^+ sensitivity and purinergic drive to RTN neurons to be ~1.5 and ~1.3, respectively. *C*, purinergic drive to chemosensitive RTN neurons shown as % inhibition of CO_2/H^+ sensitivity by PPADS ($n = 11$) or suramin ($n = 11$) at 22°C and 35°C. Note that increasing temperature did not increase the purinergic drive to chemosensitive RTN neurons.


A
Suramin
CO2
CO2
CO2
CO2
CO2
Firing Rate (Hz)
4
3
2
1
0
5 min
°C
35
22
bath temperature
B
22°C
35°C
35°C + drug
CO2-induced
Δ Firing Rate (Hz)
4
2
0
PPADS
Suramin
C
100
22°C
35 °C
% Inhibition
0
PPADS
Suramin

by 2.38 ± 0.23 Hz (n = 8); this response was significantly less than the CO_2/H^+ response at 35°C in the absence of the P2 receptor antagonist (initial response or after wash out) (Fig. 8*A*–*B*). This purinergic-sensitive component of RTN chemoreception has an estimated Q_{10} of ~1.3. It is worth noting that this value is similar to that described for connexin-mediated ATP release or current flux (Q_{10} = 1.2–1.4; (Bukauskas, Elfgang et al. 1995, Valiunas, Manthey et al. 1999, Leybaert, Braet et al. 2003). In addition, heating from 22°C to 35°C did not significantly increase effects of P2 receptor antagonists on CO_2/H^+ sensitivity of RTN neurons; PPADS and suramin decreased chemoreceptor activity by 27.3 ± 4.8% (n = 12) and 27.9 ± 4.5% (n = 8) at 22°C and 35°C, respectively (Fig. 8*C*). These results indicate that purinergic drive to CO_2/H^+-sensitive RTN neurons does not vary over this temperature range; therefore, all subsequent *in vitro* experiments were performed at room temperature.

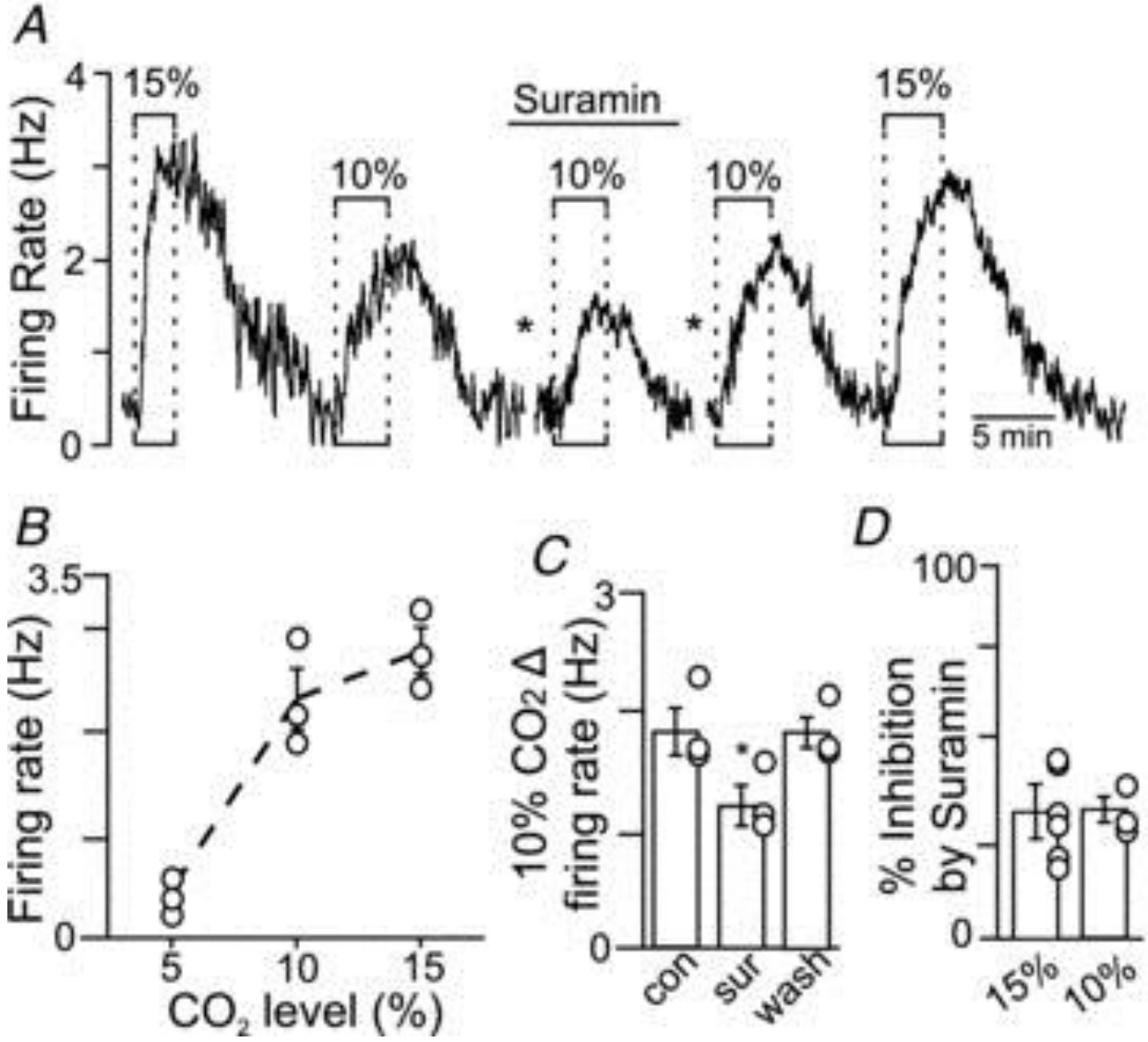
To address the possibility that purinergic drive to RTN neurons is stimulus dependent, we compared effects of suramin on the firing rate response of RTN neurons to 10% and 15% CO_2. As shown in Fig. 9*A* and *B*, exposure to 10% (pH_o 7.10) and 15% CO_2 (pH_o 6.90) increased chemoreceptor activity by 1.83 ± 0.17 Hz and 2.48 ± 0.20 Hz (n = 3), respectively. In addition, suramin had similar effects on the firing rate response of RTN neurons to 10% and 15% CO_2. Previous (Wenker, Kreneisz et al. 2010) and current results (Fig. 9*C* and *D*) show that ~10 min incubation in suramin (100 μm) decreased the response of RTN neurons to 10% and 15% CO_2 by 33.5 ± 3.3% and 31.9 ± 7.2%, respectively (Fig. 9*C* and *D*), suggesting that purinergic drive to these cells is relatively constant over this CO_2 range.

Changes in the extracellular Ca^{2+} gradient do not affect purinergic drive to chemoreceptors

Evidence suggests that H^+-evoked ATP release triggers Ca^{2+} influx into ventral surface astrocytes, possibly via P2X receptors, and this leads to bulk ATP release and subsequent activation of CO_2/H^+-sensitive RTN neurons (Gourine, Kasymov et al. 2010)). To explore this possibility further, we tested the effects of a high $MgCl_2$ and low $CaCl_2$ medium on the CO_2/H^+ sensitivity of RTN neurons. We used low (200 μm) rather than zero Ca^{2+} in order to minimize the effects of low Ca^{2+} on gap junctions. In addition,

Figure 9

Purinergic drive does not increase with increased CO_2 intensity. *A*, firing rate trace shows that exposure to 10 and 15% CO_2 increased neuronal activity by 2.9 and 2.1 Hz, respectively. In the presence of suramin (100 μm), exposure to 10% CO_2 increased neuronal activity only 1.6 Hz. Responsiveness to 10 and 15% CO_2 was fully recovered when suramin was washed out. The asterisks denote 10 min time breaks. *B*, average data (n = 3) show that exposure to 10 and 15% CO_2 increased neuronal activity in a relatively linear manner. *C*, summary data (n = 3) show that suramin decreased the firing rate response to 10% CO_2. *D*, summary data (n = 3) show that suramin decreased the responsiveness to 10 and 15% CO_2 by similar amounts, indicating that purinergic drive to chemosensitive RTN neurons does not increase in response to this range of stimulus intensities.


A
Firing Rate (Hz)
4
2
0
15%
10%
Suramin
10%
10%
15%
5 min
B
Firing rate (Hz)
3.5
0
5
10
15
CO2 level (%)
C
10% CO2 Δ firing rate (Hz)
3
0
con
sur
wash
D
% Inhibition by Suramin
100
0
15%
10%

this medium has been shown to effectively block Ca^{2+}-dependent exocytosis with little disruption of intrinsic membrane characteristics (Richards, Sercombe 1970). As before, CO_2/H^+-sensitive RTN neurons were identified based on their robust firing rate response to 15% CO_2 in normal Ringer solution. Exposure to low Ca^{2+}–high Mg^{2+} medium alone did not consistently increase baseline activity, suggesting that this low level of extracellular Ca^{2+} did not facilitate gap junction-mediated ATP release. However, as noted below low Ca^{2+}–high Mg^{2+} medium can inhibit excitatory input to RTN chemoreceptors (Fig. 10*C*), and so potentially offset excitatory effects of connexin-mediated ATP release. A second exposure to 15% CO_2, this time after ~10 min incubation in low Ca^{2+}–high Mg^{2+} solution, also elicited a strong firing rate response (Fig. 10*A* and *B*), indicating that a 10-fold decrease in the extracellular Ca^{2+} gradient did not affect CO_2/H^+ sensitivity (Fig. 10*B*). To confirm that Ca^{2+}-dependent exocytosis was effectively attenuated by these incubation conditions, we recorded excitatory post-synaptic currents (EPSCs) from RTN chemoreceptor neurons during exposure to the low Ca^{2+}–high Mg^{2+} solution. As expected, ~5 min perfusion with low Ca^{2+}–high Mg^{2+} solution caused a robust and reversible decrease in EPSC frequency with only a modest effect on amplitude (Fig. 10*C–E*). These results suggest that the CO_2/H^+ sensitivity of RTN neurons, including the purinergic component, is not dependent on neuronal vesicle release.

Gap junction hemichannels contribute to CO_2/H^+-evoked ATP release in the RTN

There is evidence that connexin 26 hemichannels are directly gated by CO_2 (Huckstepp, Eason et al. 2010) and contribute to CO_2-evoked ATP release in the RTN (Huckstepp, id Bihi et al. 2010). To test whether connexin-mediated transmitter release contributes to the CO_2/H^+ sensitivity of RTN neurons, we used CBX and cobalt to block hemichannels. We found that ~10 min incubation in CBX (100 μm) decreased neuronal CO_2/H^+ sensitivity by 0.74 ± 0.18 Hz (n = 6) or 27.7 ± 5.5% (Fig. 11*A* and *B*). Likewise, incubation in cobalt (500 μm) decreased neuronal CO_2/H^+ sensitivity by 0.64 ± 0.08 Hz (n = 5) or 26.36 ± 2.73% (Fig. 11*C* and *D*). Note that both gap junction blockers decreased neuronal CO_2/H^+ sensitivity by an amount similar to the previously reported effects of P2 receptor antagonists (PPADS and suramin); at room temperature P2 receptor antagonists decreased CO_2/H^+ sensitivity of RTN neurons by

Figure 10

Purinergic drive to RTN neurons is not dependent on extracellular Ca^{2+}. *A*, firing rate trace shows that the CO_2/H^+ sensitivity of an RTN neuron was unaffected by low Ca^{2+}–high Mg^{2+} synaptic block (SB) medium. The asterisks denote 8 min time breaks. *B*, average data (n = 5) show that SB medium did not significantly affect CO_2/H^+ sensitivity (P = 0.982). *C*, traces of holding current (at a potential of −60 mV) show that exposure to SB medium blocked spontaneous excitatory post-synaptic currents (EPSCs) in chemosensitive RTN neurons. *D* and *E*, as expected, SB medium inhibited EPSC frequency (*D*, n = 3) but not EPSC amplitude (*E*, n = 3). Average decay time constant was 4.8 ± 0.2 ms (not shown). These results suggest that extracellular Ca^{2+} is not required for the purinergic drive to breathe.

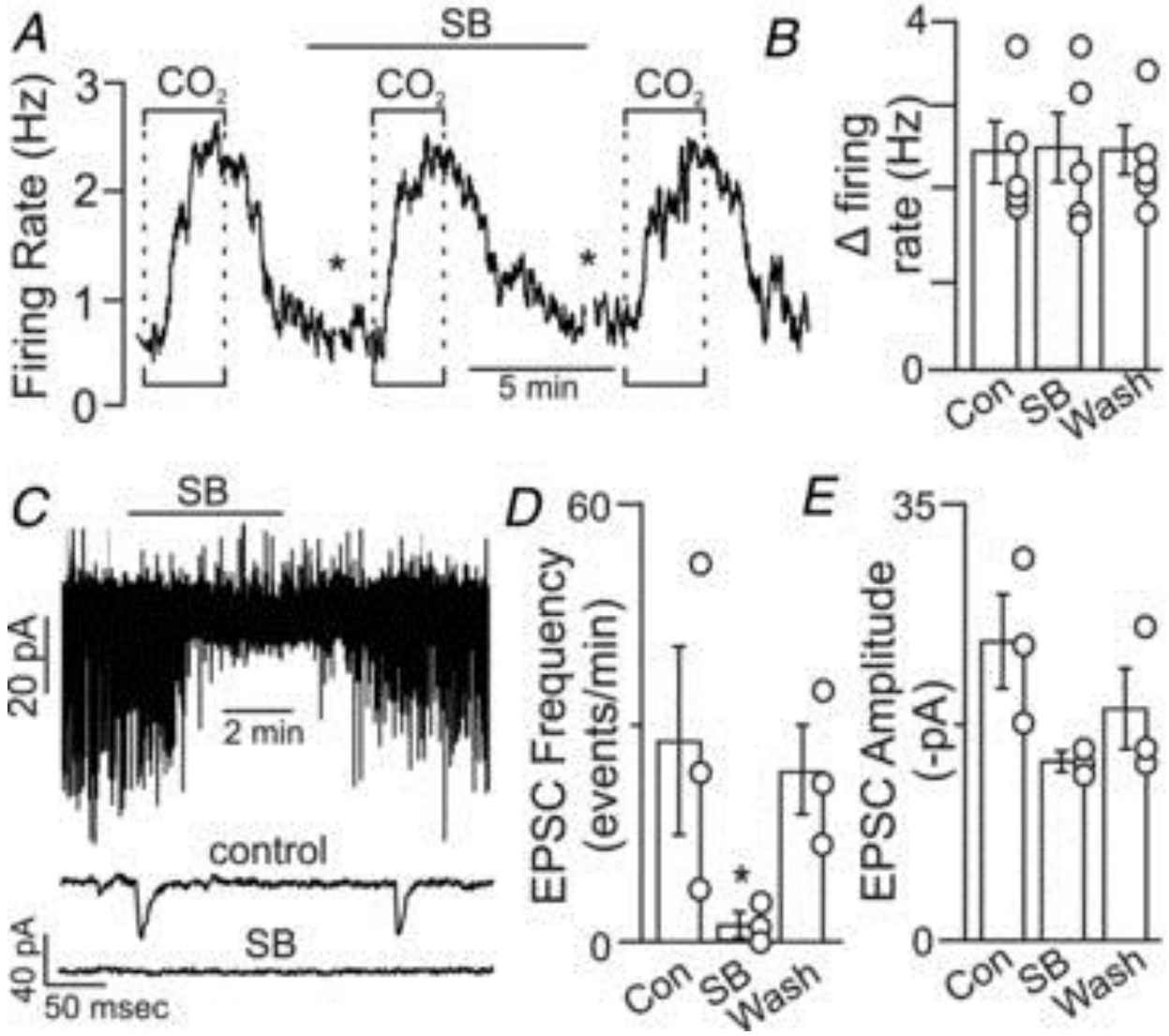
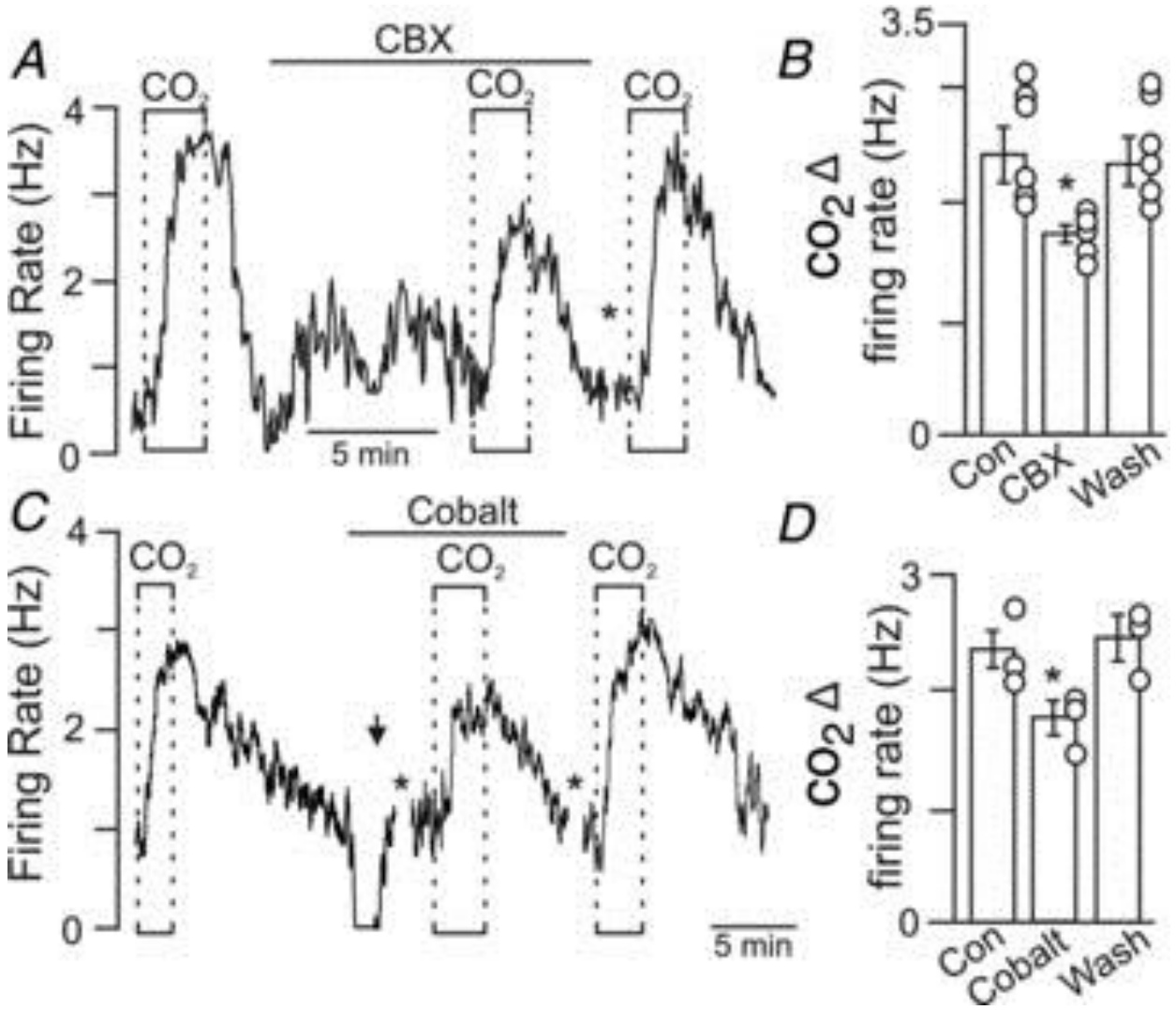

A
SB
CO2
CO2
CO2
Firing Rate (Hz)
3
2
1
0
5 min
B
Δ firing rate (Hz)
4
0
Con
SB
Wash
C
SB
20 pA
2 min
control
SB
40 pA
50 msec
D
EPSC Frequency (events/min)
60
0
Con
SB
Wash
E
EPSC Amplitude (-pA)
35
0
Con
SB
Wash

Figure 11

Gap junction blockers decrease purinergic drive to RTN neurons. *A*, trace of firing rate shows that under control conditions, exposure to 15% CO_2 increased the neuronal activity ~3 Hz. A second exposure to 15% CO_2, this time after 10 min incubation with carbenoxolone (CBX, 100 μm), increased firing rate only ~2 Hz. The asterisk indicates a gap in recording during the CBX wash. *B*, average data (n = 6) show that CBX decreased CO_2/H^+ sensitivity by ~30%. *C*, trace of firing rate shows that exposure to 15% CO_2 increased neuronal activity 2.1 Hz. After returning to control conditions, exposure to cobalt (500 μm) inhibited neuronal activity; therefore a depolarizing current (5 nA; arrow) was injected into the cell to approximate baseline activity under control conditions. In the continued presence of cobalt (with a holding current of 5.0 nA), a second exposure to 15% CO_2 this time only increased firing rate ~1.5 Hz. CO_2 responsivness was fully recovered after washing out cobalt for ~10 min. *D*, average data (n = 5) show that cobalt decreased CO_2/H^+ sensitivity by ~30%.


A
CBX
CO2
CO2
CO2
Firing Rate (Hz)
4
2
0
5 min
B
3.5
CO2 Δ firing rate (Hz)
0
Con
CBX
Wash
C
Cobalt
CO2
CO2
CO2
Firing Rate (Hz)
4
2
0
5 min
D
3
CO2 Δ firing rate (Hz)
0
Con
Cobalt
Wash

0.7 ± 0.18 Hz or 27.3 ± 4.8% (Wenker, Kreneisz et al. 2010). Furthermore, in combination suramin and cobalt decreased CO_2/H^+ sensitivity of RTN neurons by 28 ± 3.0% (Fig. 12*A* and *B*). The per cent inhibition by suramin and cobalt acting together was not different from that of either blocker alone (Fig. 12*C*), further suggesting that the majority of CO_2-evoked ATP release is mediated by gap junction hemichannels. Together, these results strongly suggest that gap junction hemichannels mediate CO_2-evoked ATP release in the RTN.

P2Y1 receptors in the RTN do not contribute to central chemoreception *in vitro* or *in vivo*

Evidence suggests that P2Y receptors mediate the excitatory effects of ATP on CO_2/H^+-sensitive RTN neurons (Mulkey, Mistry et al. 2006, Wenker, Kreneisz et al. 2010). Of these, P2Y1 receptors have been shown to function as the primary substrate for ATP-mediated activation of inspiratory neurons in the preBötzinger complex (Lorier, Lipski et al. 2008, Lorier, Huxtable et al. 2007), a region critically involved in inspiratory rhythm generation (Smith, Ellenberger et al. 1991). Therefore, we considered the possibility that P2Y1 receptors also contribute to up-stream purinergic modulation of CO_2/H^+-sensitive RTN neurons. To make this determination, we tested effects of MRS2179 (a potent and specific P2Y1 receptor blocker) on CO_2 sensitivity of RTN neurons *in vitro* and *in vivo*. In the brain slice preparation, incubation (~10 min) in MRS2179 (3 μm) had negligible effects on baseline activity and CO_2/H^+ sensitivity of RTN neurons (Fig. 13*A* and *B*). In addition, focal application of a specific P2Y1 receptor agonist (MRS2365, 100 μm) had no effect on activity of CO_2/H^+-sensitive RTN neurons (Fig. 13*A*). These results suggest P2Y1 receptors are not expressed by CO_2/H^+-sensitive RTN neurons and do not contribute to their CO_2 sensitivity.

To determine if P2Y1 receptors contribute to CO_2-induced changes in breathing or blood pressure *in vivo*, we measured CO_2 responsivness after bilateral RTN injections of saline or MRS2179. As described above, under control conditions (i.e. after saline injections), exposure to hypercapnia (10% CO_2) increases PNA and causes hypotension (−16 ± 6 mmHg) followed by an increase in MAP (24 ± 5 mmHg) (Figs 7

Figure 12

In combination, P2 receptor antagonist and gap junction blockers decrease CO_2/H^+ sensitivity by an amount similar to each blocker alone. *A*, trace of firing rate shows that under control conditions exposure to 15% CO_2 increased neuronal activity ~2 Hz. A second exposure to 15% CO_2, this time in the presence of suramin to block P2 receptors and cobalt to block gap junction hemichannels, increased firing rate only ~1 Hz or 72% of control. The asterisk indicates an 8 min time break. CO_2 sensitivity fully recovered after washing out suramin and cobalt. *B*, summary data (n = 3) show that in combination suramin and cobalt decreased CO_2/H^+ sensitivity of RTN neurons by 28 ± 3.0%. *C*, the per cent inhibition by suramin and cobalt acting together is not different from that of either blocker alone.

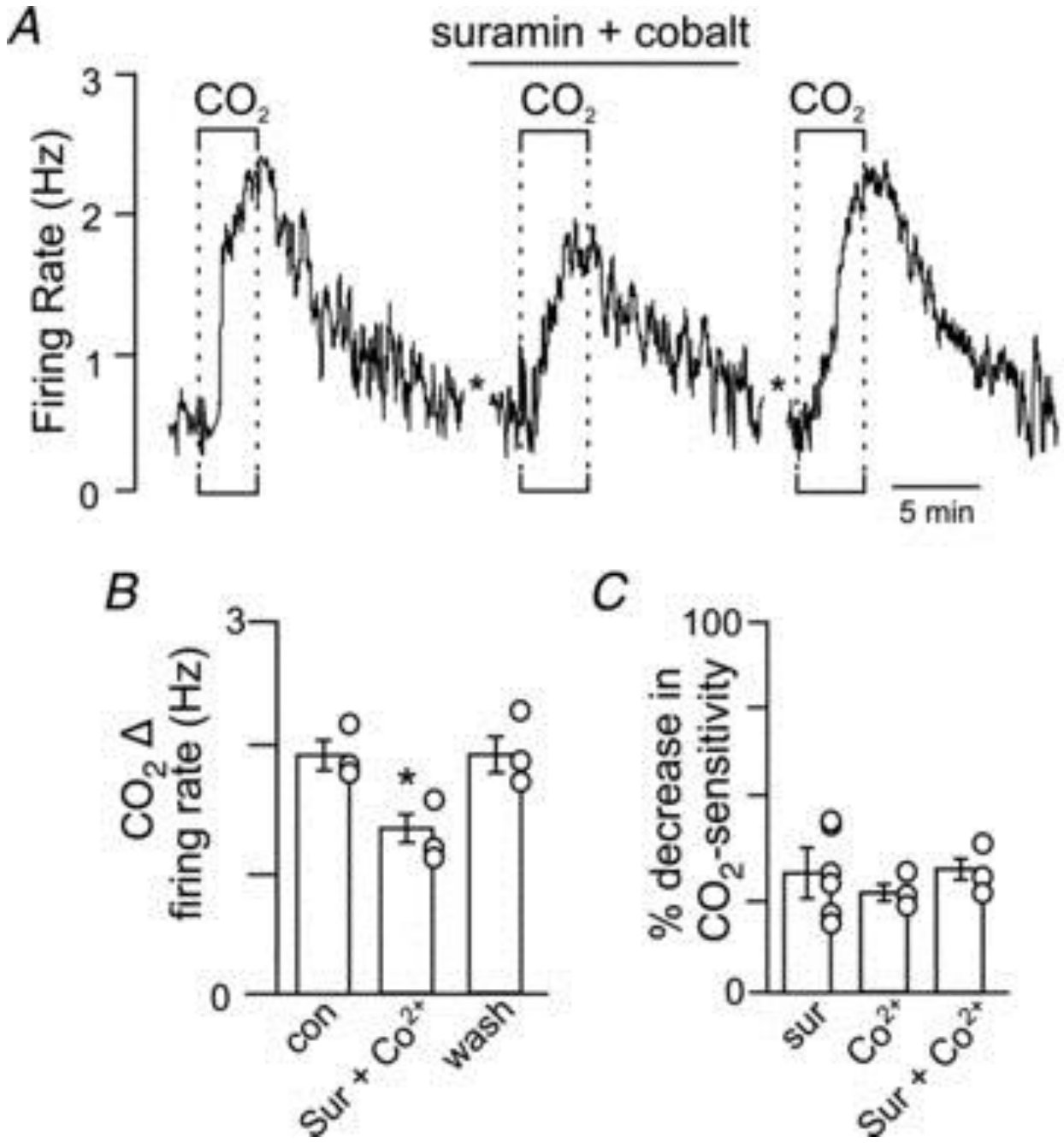
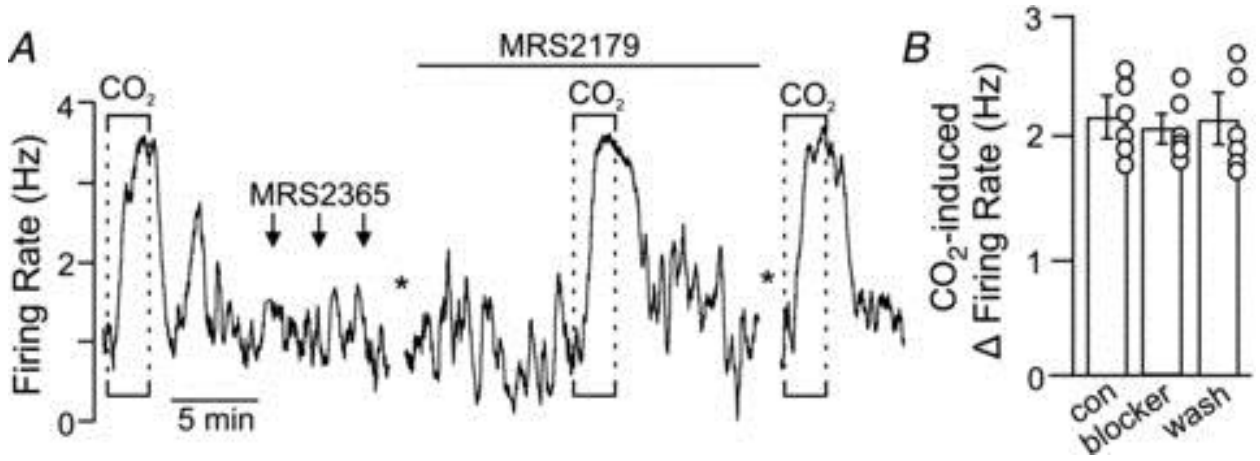

A
suramin + cobalt
CO2
CO2
CO2
Firing Rate (Hz)
3
2
1
0
5 min
B
CO2 Δ firing rate (Hz)
3
0
con
Sur + Co2+
wash
C
% decrease in CO2-sensitivity
100
0
sur
Co2+
Sur + Co2+

Figure 13

Purinergic drive to RTN neurons does not depend on P2Y1 receptors. *A*, trace of firing rate shows that under control conditions exposure to 15% CO_2 increased neuronal activity ~3 Hz. A second exposure to 15% CO_2, this time in the presence of the P2Y1 receptor-specific antagonist MRS2179 (3 μm), also increased firing rate by ~3 Hz. The asterisk indicates a 10 min time break during which the tissue was incubated in MRS2179. Arrows indicate when the P2Y1-specific agonist MRS2365 (100 μm in 7.3 Hepes buffer) was focally applied. *B*, summary data (n = 6) show that MRS2179 had no effect on the CO_2/H^+ sensitivity of RTN neurons.


A
MRS2179
CO2
CO2
CO2
MRS2365
Firing Rate (Hz)
4
2
0
5 min
*
*
B
CO2-induced
Δ Firing Rate (Hz)
3
2
1
0
con
blocker
wash

and 14). Bilateral RTN injections of MRS2179 (100 μm in 50 nl) did not change resting MAP (119 ± 5 mmHg compared with saline 121 ± 3 mmHg) or baseline PNA activity (102 ± 5% of control). In addition, injections of MRS2179 did not affect the CO_2-induced pressor response (24 ± 5 mmHg *vs*. saline 19 ± 4 mmHg) or the CO_2-induced increase in PNA amplitude (86 ± 15% of control) and PNA frequency (108 ± 5% of the control) (Fig. 14*A* and *C–E*). However, unilateral RTN injections of MRS2365 (100 μm in 50 nl) increased MAP by 23 ± 1 mmHg (compared with 4 ± 1 mmHg in control experiments) and increased PNA amplitude and frequency by 59 ± 6% and 53 ± 5%, respectively (Fig. 15*C–E*). Further, bilateral injections of MRS2179 entirely blocked the effects of MRS2365 (Fig. 15*A–E*), thus confirming effectiveness of the antagonist. These results are consistent with our *in vitro* evidence that P2Y1 receptors are not expressed by CO_2/H^+-sensitive RTN neurons and do not contribute to central CO_2 sensitivity.

DISCUSSION

There is compelling evidence that CO_2-evoked ATP release from ventral surface astrocytes contributes to respiratory drive by activating chemosensitive RTN neurons (Gourine, Kasymov et al. 2010, Gourine, Llaudet et al. 2005, Wenker, Kreneisz et al. 2010). Here, we demonstrate *in vivo* that blocking P2 receptors in the RTN lessened the changes in PNA amplitude, PNA frequency and pressor responses elicited by central chemoreflex activation. Additionally, we show *in vitro* that bath application of P2 receptor antagonists (PPADS and suramin) blunted the firing rate response of RTN neurons to 10% and 15% CO_2 by 30%. We show that the contribution of purinergic signalling to chemosensitive RTN neuronal activity is independent of temperature and stimulus strength. These results clearly indicate that purinergic signalling contributes in part to the integrated output of the RTN during hypercapnia. We also found that the purinergic drive to chemosensitive RTN neurons was wholly retained in low Ca^{2+} synaptic block medium but was significantly attenuated by gap junction blockers (CBX and cobalt), suggesting that astrocytes release ATP via gap junction hemichannels. Our results also show that P2Y1 receptors do not contribute to CO_2/H^+ sensitivity of the RTN; however, P2Y1 receptors are expressed in the region and their activation can influence respiratory and sympathetic outflow of the RTN. The identity and function

Figure 14

P2Y1 receptors do not contribute to CO_2-induced changes in breathing or blood pressure. *A*, traces of end-expiratory CO_2 ($etCO_2$), arterial pressure (AP) and integrated PNA (iPNA) show CO_2 responses 10 min after RTN injections of saline or MRS2179 (100 μm, 50 nl each side). *B*, computer-assisted plot of the centre of injection sites (Bregma −11.6; Paxinos & Watson, 1998). Scale bar in *B*, 1 mm. *C–E*, summary data (n = 5) showing changes in mean arterial pressure (ΔMAP) (*C*), PNA amplitude (ΔPNA ampl) (*D*) and PNA frequency (ΔPNA freq) (*E*) elicited by stepping the end-expiratory CO_2 from 5 to 10% after RTN injections of saline or MRS2179. Differences are expressed as a percentage of the response to CO_2 after saline injection. Abbreviations: py, pyramid; RPa, raphe pallidus; Sp5, spinal trigeminal tract; VII, facial motor nucleus.

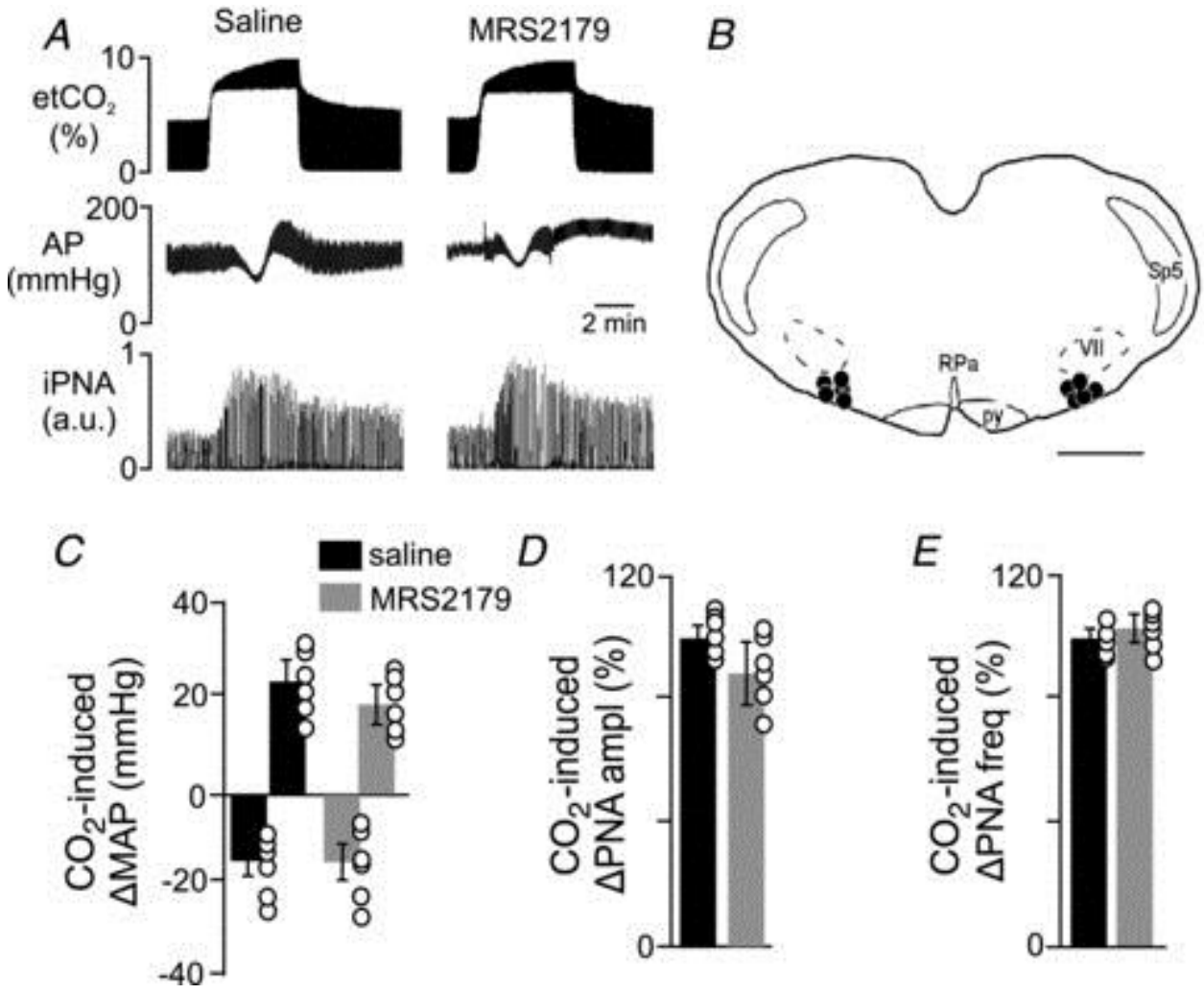
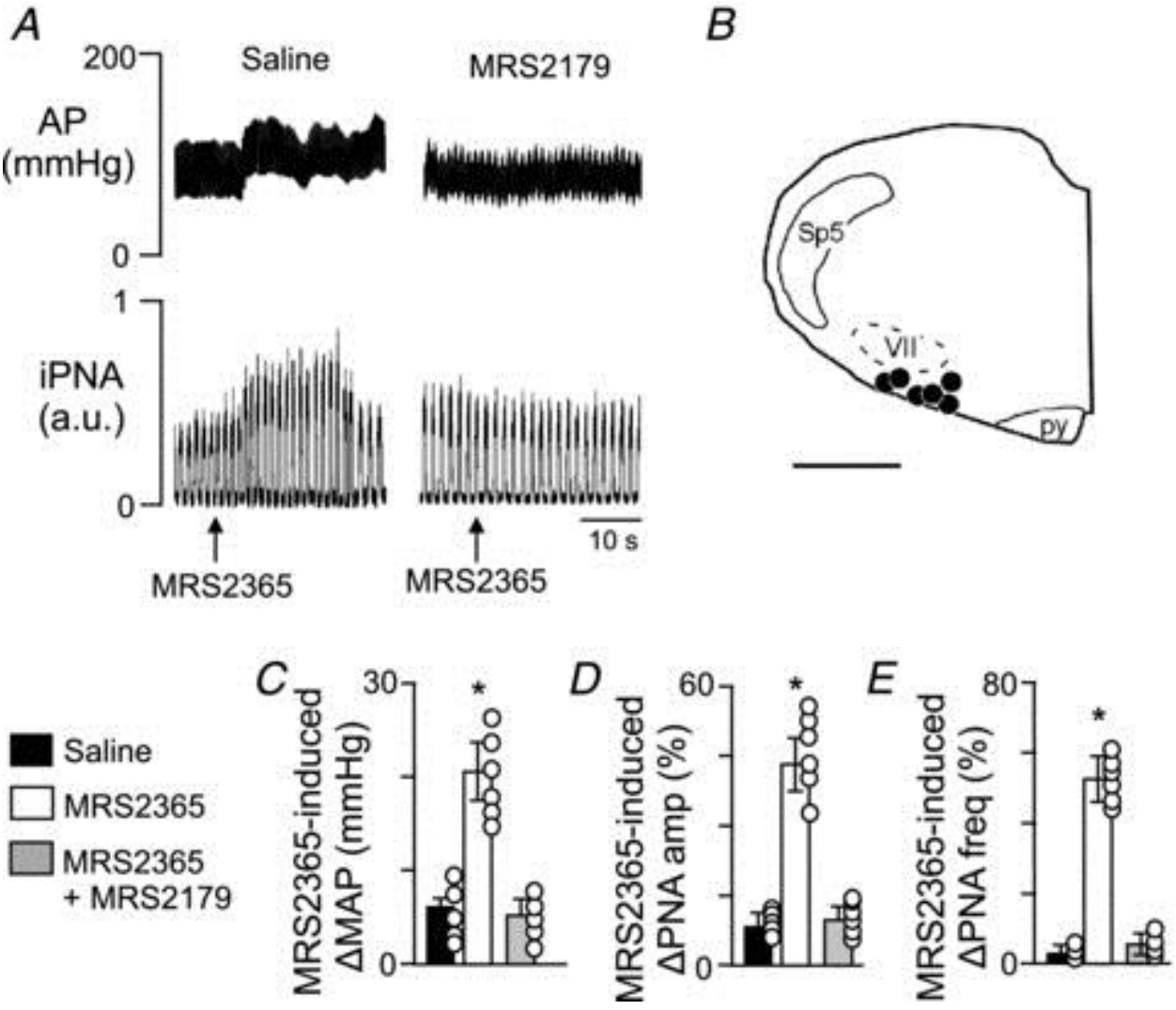

A
Saline
MRS2179
10
etCO2
(%)
0
200
AP
(mmHg)
0
2 min
1
iPNA
(a.u.)
0
B
Sp5
RPa
VII
py
C
saline
MRS2179
40
20
0
-20
-40
CO2-induced
ΔMAP (mmHg)
D
120
0
CO2-induced
ΔPNA ampl (%)
E
120
0
CO2-induced
ΔPNA freq (%)

Figure 15

RTN injections of a specific P2Y1 agonist increased arterial pressure and PNA in anaesthetized vago-sino-aortic-denervated rats. *A*, traces of arterial pressure (AP) and integrated phrenic nerve activity (iPNA) show that unilateral RTN injections of the specific P2Y1 receptor agonist MRS2365 (100 μm in 50 nl) increased AP and PNA. *B*, computer-assisted plot of injection sites (coronal plane at Bregma −11.6 (Paxinos & Watson, 1998). Scale bar in *B*, 1 mm. *C–E*, summary data (n = 6) show MRS2365-induced ΔMAP (*B*), ΔPNA amp (*C*) and ΔPNA freq (*D*) after RTN injections of saline or MRS2179. Differences expressed as a percentage of the response to the CO_2 challenge elicited during saline injection. Abbreviations: py, pyramid; Sp5, spinal trigeminal tract; VII, facial motor nucleus.


A
200
Saline
MRS2179
AP
(mmHg)
0
1
iPNA
(a.u.)
0
10 s
MRS2365
MRS2365
B
Sp5
VII
py
C
D
E
Saline
MRS2365
MRS2365
+ MRS2179
MRS2365-induced
ΔMAP (mmHg)
30
0
*
MRS2365-induced
ΔPNA amp (%)
60
0
*
MRS2365-induced
ΔPNA freq (%)
80
0
*

of these P2Y1-expressing neurons remain unknown. We conclude that RTN chemoreception is determined primarily by CO_2/H^+-sensitive neurons but is augmented by gap junction-mediated, CO_2-evoked ATP release most probably from astrocytes.

Experimental limitations

Our *in vivo* experiments were performed is anaesthetized rats and it is well known that anaesthetics can modify centrally mediated cardio-respiratory reflexes. Therefore, it will be important for future studies to confirm the role of purinergic signalling in central chemoreception using conscious animals. In addition, we did not measure ATP directly but rather used the activity of RTN chemoreceptors as a functional measure of ATP release. This approach is reasonable considering that CO_2-evoked ATP release in the region of the RTN is well established (Gourine, Kasymov et al. 2010, Gourine, Llaudet et al. 2005, Huckstepp, id Bihi et al. 2010) and the focus of the current study is on the purinergic drive to RTN neurons, which has received only limited attention. This study was also limited by the lack of pharmacological tools that allow for the specific manipulation of astrocytes without confounding effects on neurons. Nevertheless, the results presented here provide important direction for future experiments that take advantage of astrocyte-specific transgenic animal models, e.g. IP3-receptor type 2 (IP3R2) knockout mice (Petravicz, Fiacco et al. 2008) or the dominant negative synaptobrevin 2 model (Pascual, Casper et al. 2005), to further dissect neuron–astrocyte interactions in the context of chemoreception.

Contribution of purinergic signalling to RTN chemoreception

Bilateral injection of PPADS into the RTN of adult rats decreased the ventilatory response to CO_2 by ~30%. These results are entirely consistent with previous evidence showing that direct application of PPADS onto the exposed ventral surface also decreased the hypercapnic ventilatory response *in vivo* by up to ~30% (Gourine, Llaudet et al. 2005). In addition, we show by using brain slices from neonatal rat pups that bath application of PPADS or suramin decreased the response of functionally identified CO_2/H^+-sensitive RTN neurons by ~30% regardless of stimulus strength or bath temperature.

Considering that P2 receptor blockers had similar effects on chemosensitivity in neonatal (postnatal days 7–12) and adult rats suggests that the purinergic drive to RTN neurons does not increase over this age range. However, astrocyte are known to undergo dramatic changes during the first postnatal week, and in other brain regions it is not until ~day 4 that characteristically mature astrocytes first appear (i.e. electrically passive) (Zhou, Schools et al. 2006). In the RTN, CO_2/H^+-sensitive astrocytes exhibit an electrically passive signature and presumably these cells are the source of ATP released in this region during hypercapnia (Wenker, Kreneisz et al. 2010). Therefore, if mature astrocytes are absent from the RTN in newborn animals, than we expect CO_2/H^+ sensitivity to be determined exclusively by intrinsic neuronal properties. This is exactly what (Onimaru, Ikeda et al. 2012) demonstrated using the *in vitro* brainstem–spinal cord preparation from newborn rats; bath application of P2 receptor blockers (MRS2179 or PPADS) had no effect on CO_2 responsivness of pFRG/RTN neurons. Our results clearly show in neonatal and adult rats that CO_2-evoked ATP release contributes to a portion of the hypercapnic ventilatory response by increasing baseline activity of chemosensitive RTN neurons at any given level of CO_2. However, it remains questionable whether purinergic signalling directly contributes to the H^+-sensing mechanism of RTN neurons. In addition, the purinergic-independent component of RTN chemoreception (~70%) was retained when synaptic activity was blocked which strengthens the possibility that RTN neurons are CO_2/H^+ sensitive (Mulkey, Mistry et al. 2006).

It is well established that hypercapnia produces biphasic changes in vascular tone, i.e hypotension followed by hypertension. The initial hypotension probably results from a direct effect of CO_2/H^+ on vasculature smooth muscle cells. Compensatory activation of sympathetic activity allows arterial pressure to recover to near control levels during hypercapnia, and accounts for the increase in arterial pressure observed at the end of the hypercapnia episode (Takakura, Moreira 2011, Mulkey, Mistry et al. 2006). Application of PPADS into the RTN region decreased the sympathetic-mediated pressure response to hypercapnia, suggesting a novel role of purinergic signalling in regulation of vascular tone during hypercapnia. For example, it is possible that CO_2-evoked ATP release provides excitatory drive to the

nearby RVLM/C1 presympathetic neurons. In the presence of PPADS this excitatory drive would be reduced and so potentially decrease sympathetic output. However, this possibility remains speculative and in need of further investigation.

The identity of P2 receptors expressed by CO_2/H^+-sensitive RTN neurons remains unknown. It was shown previously that P2Y1 receptors mediate the excitatory effects of ATP on neurons in the preBötzinger complex (Lorier, Lipski et al. 2008, Lorier, Huxtable et al. 2007). However, we found that P2Y1 receptors are not expressed by chemosensitive RTN neurons and do not contribute to CO_2/H^+ sensitivity of these cells *in vitro* or to CO_2-induced changes in breathing or blood pressure *in vivo*. Therefore, P2Y1 receptors do not contribute to RTN chemoreception. However, activation of P2Y1 receptors in the RTN increased breathing and MAP, suggesting that P2Y1 receptors are expressed in the region and, when activated, can influence respiratory and sympathetic outflow. Considering that ADP not ATP is the preferential ligand for P2Y1 receptors, we speculate that ATP released during hypercapnia is not readily broken down to ADP and adenosine, thus limiting the contribution of these unknown P2Y1-expressing neurons to regulation of breathing or blood pressure. A focus of future work will be to determine the identity and function of these P2Y1-expressing RTN neurons. Nevertheless, the absence of an identifiable G-protein-coupled purinergic receptor on chemosensitive RTN neurons suggests that one or more P2X receptors may contribute to purinergic modulation of chemosensitive RTN neurons, possibly P2X1 and/or P2X3 (Gourine, Kasymov et al. 2010).

Requisite role of gap junction hemichannels in purinergic drive to RTN chemoreceptors

Our evidence that gap junction blockers (CBX and cobalt), but not synaptic block medium, decreased chemosensitive RTN neuronal activity by an amount proportional to inhibition by P2 receptor antagonists, suggests that the purinergic drive to RTN neurons is dependent on hemichannels. We consider it unlikely that communication between astrocytes via gap junctions is a major determinant of purinergic drive to neurons in this region for two reasons. First, RTN astrocytes are unusual in that they

exhibit limited dye-coupling compared with astrocytes in other brain regions (Wenker, Kreneisz et al. 2010), suggesting that these cells are not extensively gap junction coupled. In addition, previous evidence showed that cobalt preferentially blocks hemichannel current with little effect on transjunctional current through gap junctions in embryonic stem cells (Huettner, Lu et al. 2006). Our results are consistent with a recent *in vivo* study that showed that application of connexin hemichannel antagonists near the RTN inhibited CO_2-evoked ATP release and decreased the whole animal ventilatory response to CO_2 by ~25% (Huckstepp, id Bihi et al. 2010).

There is convincing evidence that CO_2-evoked ATP release in the RTN is dependent on connexin 26 (Cx26) (Huckstepp, Eason et al. 2010). The mechanism by which CO_2 influences the gating of Cx26 hemichannels is not fully understood. Evidence suggests that molecular CO_2 binds directly to Cx26 to increase the open probability of the hemichannel (Huckstepp, Eason et al. 2010). However, it has also been shown that CO_2/H^+ sensitivity of RTN astrocytes is determined, in part, by inhibition of Kir4.1–Kir5.1 channels (Wenker, Kreneisz et al. 2010), suggesting that membrane depolarization could trigger ATP release. This mechanism could be parsimonious with the current data considering that certain hemichannels exhibit voltage-dependent gating (Saez, Retamal et al. 2005). However, rat Cx26 does not demonstrate voltage dependence (Gonzalez, Gomez-Hernandez et al. 2006); thus, if Cx26 is the main conduit for ATP release from RTN astrocytes then acid-induced changes in membrane potential might not contribute. Consistent with this possibility, a recent *in vivo* study reported that Kir5.1 knockout mice exhibit normal ventilatory response to CO_2 *in vivo* (Trapp, Tucker et al. 2011), suggesting that astrocytic membrane depolarization is not required for CO_2-evoked ATP release. However, it should be recognized that Kir5.1 channels are expressed in other organs, including the kidneys, and as described by Trapp *et al*. these conventional knockout mice have profound metabolic acidosis which could conceivably interfere with this acid-sensing mechanism. Thus, the link between CO_2/H^+-induced changes in astrocytic membrane potential and ATP release remains to be definitively established.

Role of astrocytes in RTN purinergic signalling

There is general agreement that astrocytes (or more precisely glial-like cells that express astrocytic markers) are the most likely source of ATP release (Wenker, Kreneisz et al. 2010, Gourine, Kasymov et al. 2010). For example, Gourine and colleagues demonstrated, using astrocyte-specific expression of channel rhodopsin 2, a light-sensitive cation channel, that generating calcium waves in astrocytes results in ATP release that can activate RTN neurons *in vitro* and increase breathing *in vivo* (Gourine, Kasymov et al. 2010). Likewise, we have shown that fluorocitrate-mediated activation of astrocytes resulted in increased activity of chemosensitive RTN neurons by a purinergic-dependent mechanism (Wenker, Kreneisz et al. 2010). In addition, Huckstepp and colleagues showed that hypercapnia opened connexin hemichannels on glial fibrillary acidic protein (GFAP)-expressing cells (Huckstepp, Eason et al. 2010), thus suggesting that direct CO_2 gating of connexin hemichannels results in ATP release from astrocytes. We show here that CO_2 sensitivity of RTN neurons is fully retained in low Ca^{2+}–high Mg^{2+} solution, thus further suggesting that that purinergic drive to RTN neurons is not dependent on excitatory synaptic transmission.

In summary, the RTN contains a population of CO_2/H^+-sensitive neurons that sends excitatory glutamatergic projections to respiratory centres to stimulate breathing and modulate sympathetic drive to the heart and vasculature (Takakura, Moreira 2011, Moreira, Takakura et al. 2006). In addition to activation by CO_2/H^+, RTN neurons are also modulated by various neurotransmitters, including ATP (Mulkey, Mistry et al. 2006, Gourine, Kasymov et al. 2010, Wenker, Kreneisz et al. 2010). Based on previous and present results, astrocytes (but not neurons) most probably mediate CO_2-evoked ATP release by a mechanism involving gap junction hemichannels.

Chapter 6 Purinergic signaling contributes to chemoreception at the level of the retrotrapezoid neucleus but not the nucleus of the solitary tract or medullary raphe.

INTRODUCTION

Hypercapnic acidosis (i.e., high CO_2/H^+) provides the primary stimulus to breathe. Central respiratory chemoreceptors sense changes in tissue CO_2/H^+ and send excitatory drive to respiratory centers to directly regulate depth and frequency of breathing (Feldman, Mitchell et al. 2003, Huckstepp, Dale 2011). The process of chemoreception is especially important during sleep and its disruption has been associated with central sleep apnea and hypoventilation syndrome (Dempsey, Smith et al. 2012, Guyenet, Mulkey 2010). Nevertheless, despite the importance of central chemoreceptors the cellular and molecular mechanism(s) underlying this process have yet to be fully elucidated.

Several brain regions are thought to function as important sites of chemoreception including the commissural portion of the nucleus of the solitary tract (commNTS) (Dean, Bayliss et al. 1990, Li, Nattie 2002), medullary raphe (e.g., raphe pallidus, magnus and obscurus) (Hodges, Martino et al. 2004, Nattie, Li 2001, Richerson 2004, Wang, Richerson 1999) and retrotrapezoid nucleus (RTN) (Li, Nattie 2002, Wang, Shi et al. 2013, Mulkey, Stornetta et al. 2004). Of these, the mechanism(s) of chemotransduction are best characterized at the level of the RTN where neurons have been shown to sense changes in CO_2/H^+ by two independent but coordinated mechanisms: direct H^+-mediated activation of pH-sensitive neurons by inhibition of a resting K^+ conductance (Mulkey, Stornetta et al. 2004, Mulkey, Talley et al. 2007), and indirect activation by purinergic signaling most likely from CO_2/H^+-sensitive astrocytes (Gourine, Llaudet et al. 2005, Huckstepp, id Bihi et al. 2010, Mulkey, Mistry et al. 2006, Wenker, Kreneisz et al. 2010, Wenker, Sobrinho et al. 2012). Evidence also suggests that purinergic signaling is a unique feature of RTN chemotransduction. For example, it was shown *in vivo* that CO_2/H^+ facilitates ATP release only at discrete locations near the RTN (Gourine, Llaudet et al. 2005) , and RTN astrocytes, but not cortical astrocytes respond to H^+ with increased Ca^{2+}-dependent exocytosis of ATP-containing

vesicles (Kasymov, Larina et al. 2013). However, the role of purinergic signaling in putative chemoreceptor regions outside the RTN has not been thoroughly investigated.

Indeed, it remains possible that purinergic signaling also regulates neuronal activity in other putative chemosensitive regions. For example, P2-receptors are widely distributed throughout the central nervous system including in the NTS and medullary raphe (Yao, Barden et al. 2000). In addition, injections of ATP into the commNTS increased breathing (Antunes, Braga et al. 2005), whereas application of a P2-receptor blocker to the NTS blunted peripheral chemoreflex control of cardiorespiratory function (Paton, De Paula et al. 2002).. Furthermore, injection of ATP into the medullary raphe increased breathing (Cao, Song 2007), whereas injection of a P2-receptor blocker into the rostral medullary raphe blunted the ventilatory response to CO_2 of conscious rats (da Silva, Moraes et al. 2012). These results suggest that purinergic signaling may contribute to the CO_2/H^+-dependent drive to breathe at other putative chemoreceptor regions. Therefore, the main goal of this study is to determine at the cellular and system levels whether purinergic signaling contributes to CO_2/H^+-sensitivity in the commNTS and medullary raphe

To test this possibility, we recorded activity of individual CO_2/H^+-sensitive neurons in putative chemosensitive regions *in vitro*, and phrenic nerve activity (PNA) *in vivo* during exposure to hypercapnia alone and in the presence of P2 receptor blockers. To determine if cells in the regions of interest functionally express P2-receptors, we also tested responsiveness to exogenous application of ATP *in vivo* and *in vitro*. In addition, we performed a set of immunohistochemical experiments using the astrocyte specific marker aldehyde dehydrogenase (Aldh1L1) to determine the distribution of astrocytes in the commNTS, medullary raphe and RTN. We find *in vitro* that CO_2/H^+-sensitive neurons in the commNTS respond to focal ATP application with ~3 fold increase in firing rate, and *in vivo* unilateral ATP injection into this region elicited an increase in PNA by ~81%. Application of ATP into the medullary raphe had no effect on chemoreceptor activity or cardiorespiratory output. In addition, P2 receptor blockade in the

commNTS and medullary raphe had no effect on the ventilatory response to CO_2 *in vivo*, or the firing rate response to 15% CO_2 *in vitro*. Furthermore, we find that astrocytes are underrepresented in the commNTS and medullary raphe as compared to the RTN. In conjunction with previous data that purinergic signaling is a critical component of RTN chemoreception, these results indicate that purinergic signaling is a unique feature of RTN chemoreception.

METHODS

In vivo preparation

All *in vivo* experiments conformed to the guidelines approved by the University of São Paulo Animal Care and Use Committee. All *in vivo* experiments were performed in male Wistar rats weighing 250-280 g. Surgical procedures and experimental protocols were similar to those previously described (Wenker, Sobrinho et al. 2012). Briefly, general anesthesia was induced with 5% halothane in 100% O_2. A tracheostomy was made and the halothane concentration was reduced to 1.4-1.5% until the end of surgery. The femoral artery was cannulated (polyethylene tubing, 0.6 mm o.d., 0.3 mm i.d., Scientific Commodities, Lake Havasu City, Arizona, USA) for measurement of arterial pressure (AP). The femoral vein was cannulated for administration of fluids and drugs. The occipital plate was removed, and a micropipette was placed in the medulla oblongata via a dorsal transcerebellar approach for microinjection of drugs. A skin incision was made over the lower jaw for placement of a bipolar stimulating electrode, next to the mandibular branch of the facial nerve, as previously described (Wenker, Sobrinho et al. 2012). The phrenic nerve was accessed by a dorsolateral approach after retraction of the right shoulder blade. To prevent any influence of artificial ventilation on phrenic nerve activity (PNA), the vagus nerve was cut bilaterally as follows.

Upon completion of the surgical procedures, halothane was replaced by urethane (1.2 g/kg) administered slowly i.v. All rats were ventilated with 100% O_2 throughout the experiment. Rectal temperature was

maintained at 37°C. End-tidal CO_2 was monitored throughout each experiment with a capnometer (CWE, Inc, Ardmore, PA, USA) that was calibrated twice per experiment with a calibrated CO_2/N_2 mix. This instrument provided a reading of <0.1% CO_2 during inspiration in animals breathing 100% O_2 and provided an asymptotic, nearly horizontal reading during expiration. The adequacy of anesthesia was monitored during a 20 min stabilization period by testing for the absence of withdrawal responses, pressor responses, and changes in PNA to a firm toe pinch. After these criteria were satisfied, the muscle relaxant pancuronium was administered at an initial dose of 1 mg/kg i.v. and the adequacy of the anesthesia was thereafter gauged solely by the lack of increase in AP and PNA rate or amplitude to a firm toe pinch. Approximately hourly supplements of one-third of the initial dose of urethane were needed to satisfy these criteria throughout the recording period (2-3 hours).

***In vivo* recordings of physiological variables**

As previously described, mean arterial pressure (MAP), phrenic nerve activity (PNA), and end-expiratory CO_2 ($etCO_2$) were digitized with a micro1401 (Cambridge Electronic Design), stored on a computer, and processed off-line with version 6 of Spike 2 software (Cambridge Electronic Design, Cambridge, UK). Integrated phrenic nerve activity (iPNA) was obtained after rectification and smoothing (τ = 0.015s) of the original signal, which was acquired with a 30-300 Hz bandpass filter. PNA amplitude (PNA amp) and PNA frequency (PNA freq) were normalized in each experiment by assigning to each of the two variables a value of 100 at saturation of the chemoreflex (high CO_2) and a value of 0 to periods of apnea.

Brain slice preparation

All procedures were performed in accordance with National Institutes of Health and University of Connecticut Animal Care and Use Guidelines. Brainstem slices were prepared as previously described (Wenker, Sobrinho et al. 2012). Briefly, neonatal rats (7-12 days postnatal) were decapitated under ketamine/xylazine anesthesia and transverse brainstem slices (300 μm) containing the raphe pallidus (RPa) were cut using a microslicer (DSK 1500E; Dosaka, Kyoto, Japan) in ice-cold substituted Ringer

solution containing (in mM): 260 sucrose, 3 KCl, 5 $MgCl_2$, 1 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose, and 1 kynurenic acid. Slices were incubated for ~30 min at 37°C and subsequently at room temperature in normal Ringer solution (in mM): 130 NaCl, 3 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 10 glucose. Both substituted and normal Ringer solutions were bubbled with 95% O_2-5% CO_2, extracellular pH (pHo = 7.35). It should be noted that slices containing the commNTS were prepared as described above but with two exceptions: 1) slices were cut in normal Ringer solution (not substituted ringers); and 2) slices were incubated at room temperature (not 37°C) for 60 minutes or more.

Slice-patch electrophysiology

Individual slices were transferred to a recording chamber mounted on a fixed-stage microscope (Zeiss Axioskop FS) and perfused continuously (~2 ml min^{-1}) with a bath solution of normal Ringer solution (same as incubation Ringers above) bubbled with 95% O_2-5% CO_2 (pHo = 7.35). The pH of the bicarbonate-based bath solution was decreased to 6.90 by bubbling with 15% CO_2. All recordings were made with an Axopatch 200B patch-clamp amplifier, digitized with a Digidata 1322A A/D converter, and recorded using pCLAMP 10.0 software (Molecular Devices). Recordings were obtained at room temperature (~22 ºC) with patch electrodes pulled from borosilicate glass capillaries (Warner Instruments) on a two-stage puller (P89; Sutter Instrument) to a DC resistance of 4–6 MΩ when filled with an internal solution containing the following (in mM): 120 KCH_3SO_3, 4 NaCl, 1 $MgCl_2$, 0.5 $CaCl_2$, 10 HEPES, 10 EGTA, 3 Mg-ATP, 0.2% biocytin, and 0.3 GTP-Tris (pH 7.2); electrode tips were coated with Sylgard 184 (Dow Corning). All recordings of neuronal firing rate were performed in the cell-attached configuration to ensure minimal alteration of the intracellular milieu. Firing rate histograms were generated by integrating action potential discharge in 10-s bins and plotted using Spike 5.0 software. Whole cell voltage clamp recordings (Ihold = -60 mV, in tetrodotoxin to block action potentials) were made to verify the serotonergic phenotype of RPa neurons; i.e., medullary raphe neurons are known to exhibit a serotonin-activated inward potassium conductance (Bayliss, Li et al. 1997).

Drugs

All drugs were purchased from Sigma unless otherwise indicated. For *in vivo* experiments, the non-specific P2-receptor antagonist pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) was diluted to 100 μM in sterile saline (pH 7.4) or ATP (10 mM) and injected into the RTN using single-barrel glass pipettes (tip diameter of 25 μm) connected to a pressure injector (Picospritzer III, Parker Hannifin Corp, Cleveland, OH). For each injection we delivered a volume of 50 nl over a period of 5s. These glass pipettes also allowed recordings of field potential properties that were used to help direct the electrode tip to the desired site. Injections in the RTN region were guided by recordings of the facial field potential (Brown, Guyenet 1985), and were placed 250 μm below the lower edge of the field, 1.7 mm lateral to the midline, and 200 μm rostral to the caudal end of the field. Recordings were made on one side only; the second injection was made 1-2 min later at the same level on the contralateral side. Injections into the medullary raphe were placed in the raphe pallidus (N= 6) and the parapyramidal region (N=7). Injections in the commNTS region were centered ~400 μm caudal to the *calamus scriptorius*. We included a 5% dilution of fluorescent latex microbeads (Lumafluor, New City, NY) with all drug applications to mark the injection sites and to verify spread of the injections.

For *in vitro* experiments, we bath applied suramin (100 μM) to block P2-receptors. In addition, ATP (1 mM in HEPES buffered medium, pH 7.3) was delivered focally using low-resistance pipettes connected to a Picospritzer III (Parker Instrumentation, Cleveland, OH). Application times were 600 ms, and vehicle control experiments were performed to ensure agonist responses were not attributable to pressure artifacts.

Immunohistochemistry

Anesthetized rat pups (p7-12) were perfused with 4% paraformaldehyde (PFA) solution through the left ventricle. The brain was dissected and fixed overnight at 4°C in 25 ml of 4% PFA. Coronal medullary slices (50 μm thick) containing the RTN, NTS, and RPa were obtained with a Leica microslicer. Sections

were stored in cryogenic glycol solution at -20°C prior to staining. Fixed slices were rinsed three times in PBS and blocked and permeabilized in PBS solution with 5% normal goat serum (NGS) and 0.5% Triton X-100. Sections were incubated overnight for two days at 4°C in primary antibodies (mouse anti-Aldh1L1, 1:1000 dilution) diluted in 5% NGS, 0.5% Triton X-100 and PBS. Sections were rinsed 3 times in PBS and incubated in secondary antibodies (Cy3-conjugated donkey anti-rabbit IgG, 1:500 dilution; Alexa 488-conjugated donkey anti-mouse IgG, 1:1000 dilution) diluted in 5% NGS, 0.5% Triton, and PBS for one hour at room temperature. The slices were mounted using Vectashield with DAPI.

Confocal ***z***-stack images were taken of the RTN, commNTS, and raphe pallidus using a Nikon A1R confocal microscope with 40x oil immersion objective. Serial z-stacks were taken of both DAPI and alexa 488 channels, to identify nuclei and astrocyte soma a processes. The z-stacks were processed and analyzed in Image J. Astrocytes were counted by identifying nuclei with greater than 50% surrounded by Aldh1L1 staining. Volume was determined by measuring the 2-D (x-y plane) area of interest and multiplying this area by the slice thickness (z stack depth) of 50 µm. Astrocyte cell counts for each slice (N=4 animals) were normalized to volume.

Statistics

Data are reported as mean ± standard error of the mean. Statistical analysis was performed using Sigma Stat version 3.0 software (Jandel Corporation, Point Richmond, CA). T-test, paired t-test or one-way ANOVA followed by the Newman-Keuls multiple comparisons test were performed as appropriate ($p < 0.05$).

RESULTS

This study is composed of both *in vitro* and *in vivo* experiments. In the brain slice preparation, I determine if purinergic signaling can influence basal activity or CO_2/H^+-sensitivity of putative chemoreceptors by testing effects of focal ATP application on firing rate and bath application of a P2

receptor blocker (suramin or PPADS) on CO_2/H^+-responsiveness of neurons in the commNTS and medullary raphe. To provide a functional basis for these results, my collaborators at the University of Sao Paulo tested *in vivo* the effects of ATP and PPADS on baseline cardiorespiratory activity and CO_2-responsivness. As a positive control for ATP injections in the commNTS and raphe, my collaborators also test effects of focal ATP application into the RTN on breathing and blood pressure. In addition, considering that astrocytes are the most likely source of CO_2/H^+-evoked ATP release in the RTN, we performed a set of immunohistochemical experiments using the astrocyte specific marker aldehyde dehydrogenase (Aldh1L1) to determine the relative density of astrocytes in the RTN, commNTS and medullary raphe.

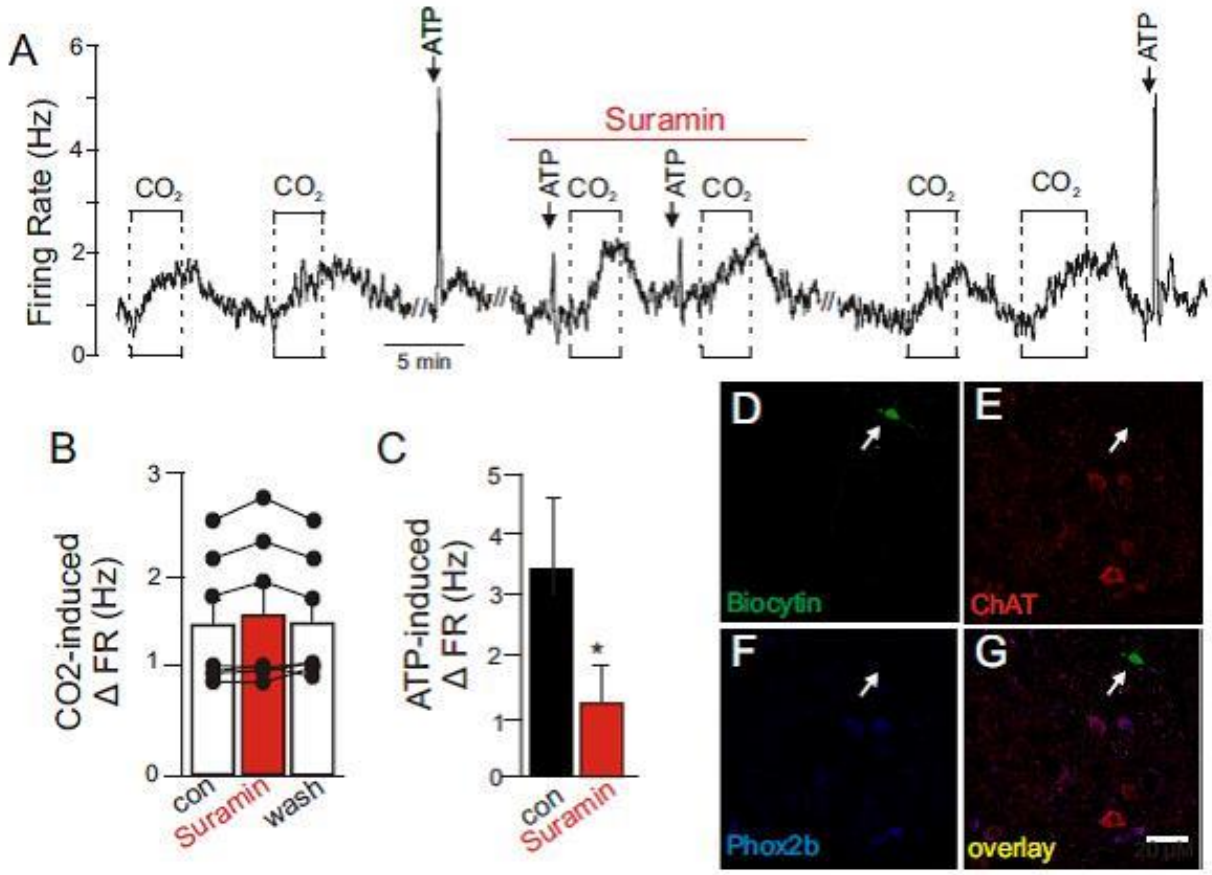
Purinergic signaling in the commNTS increases activity of chemosensitive neurons and respiratory activity but does not contribute to CO_2/H^+-responsiveness.

We recorded from CO_2/H^+-sensitive commNTS neurons in slices to characterize effects of purinergic signaling on baseline activity and CO_2/H^+-responsiveness. As previously described (Conrad, Nichols et al. 2009), a neuron was designated CO_2/H^+-sensitive if it was spontaneously active under control conditions (5% CO_2) and responded to 15% CO_2 with a $\geq$ 0.5 Hz increase in firing rate (this corresponds with a chemosensitivity index of ~150%). We did not find any CO_2-inhibited cells but this is not surprising considering that only a small minority of cells in this region have been shown to be inhibited by hypercapnia (Dean, Bayliss et al. 1990). The average firing rate of CO_2/H^+-sensitive cells was 0.58 ± 0.14 Hz (N = 7) under control condition and 2.06 ± 0.46 Hz in 15% CO_2 (i.e., CO_2 increased activity 1.49 ± 0.25 Hz, N = 7, Figs. 16A-B). This level of CO_2/H^+-sensitivity is nearly identical to what has been described previously for cells in this region (Conrad, Nichols et al. 2009, Dean, Bayliss et al. 1990) so we are confident that we are recording from the group of cells thought to function as chemoreceptors. To gain some insight into the neurochemical phenotype of CO_2-sensitive commNTS neurons, we filled recorded cells with biocytin for subsequent immunohistochemical analysis using antibodies for the transcription

factor Phox2b and choline acetyltranferase (ChAT; marker of cholinergic neurons). We found that 5 of 5 CO_2-sensitive commNTS neurons were negative for Phox2b and ChAT (Figs. 16D-G).

Figure 16

Purinergic signaling modulates activity but not CO_2/H^+-sensitivity of NTS neurons in vitro. A, firing rate trace from a commNTS neuron shows that under control conditions exposure to 15% CO_2 increased neuronal activity of ~1 Hz in a reversible and repeatable manner. Under control conditions, focal application of ATP (1 mM) evoked a rapid and robust increase in firing rate of ~ 3 Hz. Bath application of the P2-receptor blocker suramin (100 μM) had no effect on basal activity but significantly attenuated the firing rate response to ATP. In the continued presence additional bouts of 15% CO2 increased firing rate by an amout similar to under control conditions. **B,** summary data (N=5) shows that suramin had no measurable effect on CO_2/H^+-sensitivity of commNTS neurons. **C,** summary data (N=5) confirms that suramin effectively blunted ATP responsiveness of these neurons. * different from control ($p < 0.05$). **D-G**, biocytin labeled CO_2/H^+-sensitive commNTS neuron (**D**, arrow) is immuno-negative for choline acetyltranferase (ChAT) (**E**) and phox2b (**F**), panel G shows merged images of the same cell in D-F. Each image is the average projection of a confocal stack; around 30 images taken every 0.26 μm in the *Z* plane. Scale bar is 20 μm.


A
Firing Rate (Hz)
6
4
2
0
ATP
Suramin
CO2
CO2
ATP
CO2
ATP
CO2
CO2
CO2
ATP
5 min
B
CO2-induced
Δ FR (Hz)
3
2
1
0
con
Suramin
wash
C
ATP-induced
Δ FR (Hz)
5
4
3
2
1
0
*
con
Suramin
D
Biocytin
E
ChAT
F
Phox2b
G
overlay

To determine if purinergic signaling contributes to activity of commNTS neurons, we first measured the firing rate response CO_2/H^+-sensitive neurons in this region to focal application of ATP (1 mM in HEPES buffered saline, pH 7.3). Under control conditions all five cells tested exhibited a large reversible and repeatable increase in activity (increase of 3.38 ± 1.24 Hz, N = 5) in response to ATP (Figs. 16A,C). Bath application of the P2-receptor blocker suramin had no discernible effect on baseline activity (firing rate under control conditions and in suramin was 0.58 ± 0.14 and 0.66 ± 0.13 Hz, respectively), suggesting these cell do not receive purinergic drive under control conditions. As expected, suramin decreased the firing rate response to ATP by 67.0 ± 7.2% (N = 5), thus confirming that P2-recepotrs are effectively blocked under these conditions. However, suramin did not blunt the firing rate response to CO_2 (firing rate under control conditions and in suramin was 1.49 ± 0.25 and 1.58 ± 0.28 Hz, respectively) (Figs. 16A-B). This indicates that CO_2-sensitive commNTS neurons are activated by purinergic signaling; however, this signaling mechanism does not contribute to CO_2-responsivness of these cells *in vitro*.

It is possible that endogenous purinergic drive to CO_2/H^+-sensitive commNTS neurons was disrupted in our *in vitro* preparation. Therefore, we also tested effects of commNTS injections of ATP and a P2-receptor blocker (PPADS) on baseline breathing and the ventilatory response to CO_2 in anesthetized rats. Consistent with our slice data, we found that injection of ATP (10 mM - 50 nl) into the commNTS elicited an increase in phrenic nerve amplitude (PNA ampl) by 46 ± 6% and frequency (PNA freq) by 103 ± 11%. ATP injections into this region also increased MAP (21 ± 4, vs. saline: 3 ± 1 mmHg, p = 0.0032) (Figs. 17A-E). Also consistent with our slice data, bilateral injections of a P2-receptor blocker (PPADS; 3 mM - 50 nl; N = 6/group) into the commNTS did not change resting PNA (99 ± 7% of control, p = 0.176) or MAP (124 ± 5 mmHg compared to saline 123 ± 7 mmHg, p = 0.0752) (Fig. 18A). Furthermore, bilateral PPADS injections into the commNTS did not affect cardiorespiratory response to CO_2; exposure to 10% CO_2 increased PNA amplitude (103 ± 9% vs. saline: 99 ± 13%, p = 0.175), PNA frequency (107 ± 6% vs. saline: 105 ± 4%, p = 0.156) and blood pressure (18 ± 7 mmHg vs. saline: 21 ± 4 mmHg, p = 0.136) (Figs. 18A-E).

Figure 17

Injection of ATP into the commNTS increased breathing and blood pressure in vago-sino-aortic denervated rats by a P2-receptor dependent mechanism. A, recordings of AP and PNA show that application of ATP (10 mM, 50 nl) into the commNTS increased breathing and blood pressure when preceded by injection of saline into this same region (i.e., control condition) but not after injection of PPADS into this region. Cardiorespiratory responses to ATP partly recovered after washing PPADS for ~ 1 hr. **B**, histology section showing the distribution of fluorescent microbeads within the commNTS. **C-E**, summary data (N=5) show changes in mean arterial pressure (ΔMAP) (C), PND amplitude (ΔPND ampl) (D) and PND frequency (ΔPND freq) (E) elicited by injection of saline or ATP into the commNTS. Abbreviations: Gr, gracile nucleus; cc, central canal. Scale bar in B is 100 μm.

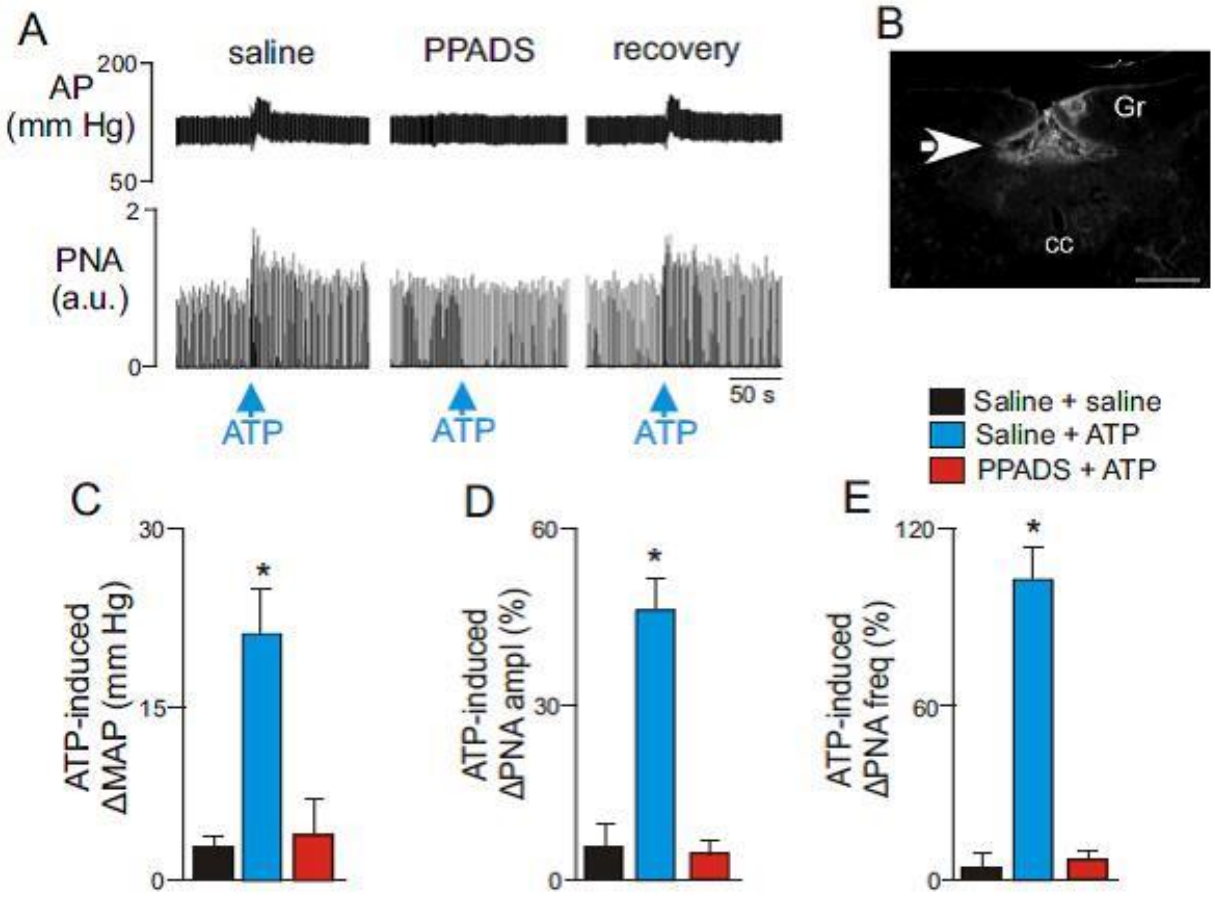
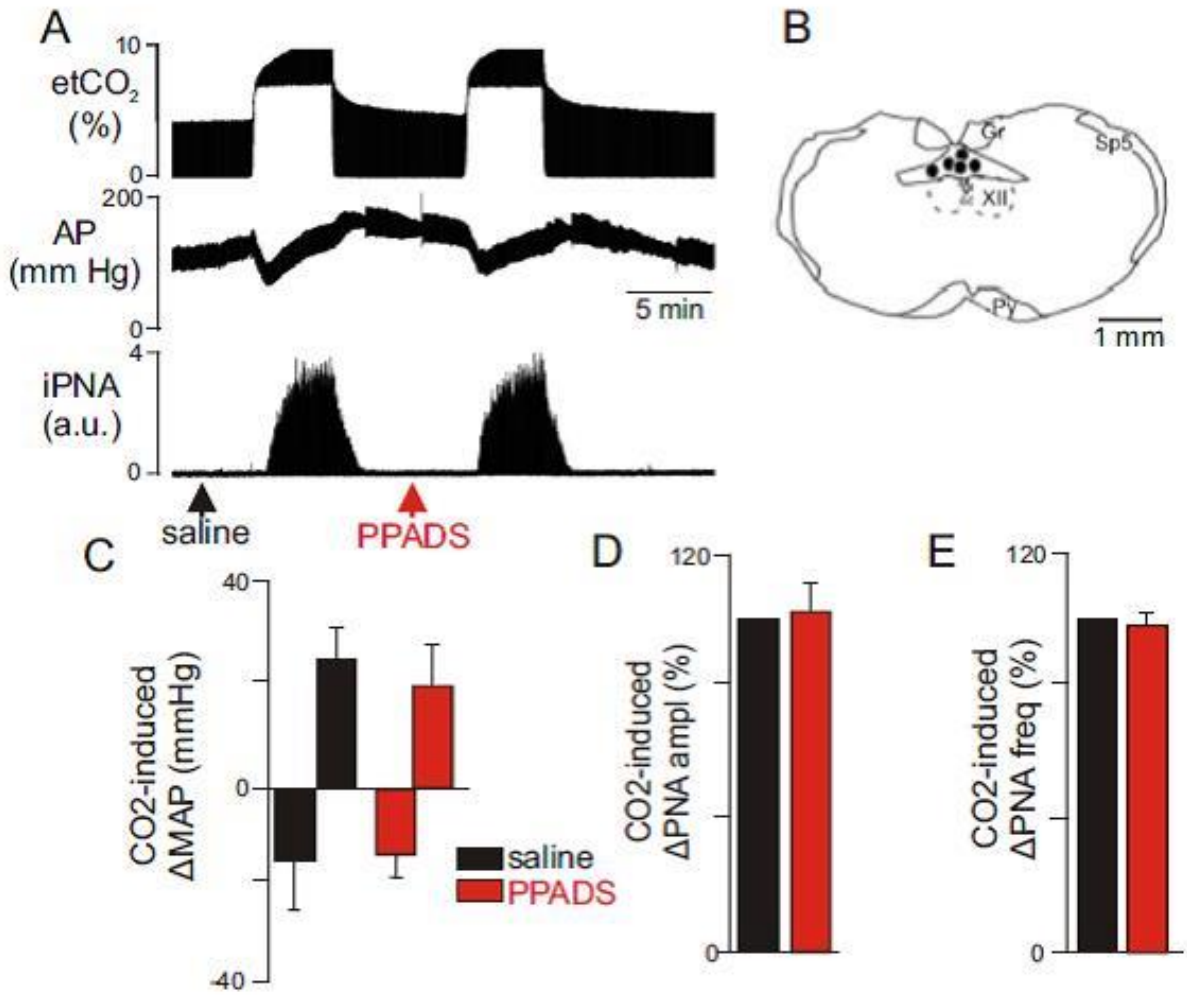

A
saline
PPADS
recovery
AP
(mm Hg)
200
50
PNA
(a.u.)
2
0
50 s
ATP
ATP
ATP
B
Gr
cc
Saline + saline
Saline + ATP
PPADS + ATP
C
ATP-induced
ΔMAP (mm Hg)
30
15
0
*
D
ATP-induced
ΔPNA ampl (%)
60
30
0
*
E
ATP-induced
ΔPNA freq (%)
120
60
0
*

Figure 18

P2-receptor blockade at the level of the commNTS had no effect on hypercapnia-induced changes in cardiorespiratory activity of anesthetized vago-sino-aortic denervated rats. A, recordings of end-expiratory CO_2 ($etCO_2$), arterial pressure (AP) and integrated phrenic nerve activity (iPNA) show the pressure and ventilatory responses of a rat to hypercapnia under control conditions (i.e., after saline injection) and 10 min after PPADS (3 mM, 50 nl) was injected into the commNTS. **B**, computer-assisted plots of the center of the injection sites revealed by the presence of fluorescent microbeads included in the injectate (coronal projection on plane Bregma -14.3 mm; (Paxinos, Watson 1982). **C-E**, summary data (N=6) show that commNTS injections of PPADS had no effect on CO2-induced changes in mean arterial pressure (ΔMAP) (C), PND amplitude (ΔPND ampl) (D) and PND frequency (ΔPND freq) (E). **Abbreviations:** Gr, gracile nucleus; py, pyramids; Sp5, spinal trigeminal tract; XII, hypoglossal motor nucleus; cc, central canal. Black dots represent the injections sites in the commNTS.


A
10
etCO2
(%)
0
200
AP
(mm Hg)
0
5 min
4
iPNA
(a.u.)
0
saline
PPADS
B
Gr
Sp5
XII
Py
1 mm
C
40
0
-40
CO2-induced
ΔMAP (mmHg)
saline
PPADS
D
120
0
CO2-induced
ΔPNA ampl (%)
E
120
0
CO2-induced
ΔPNA freq (%)

For these experiments, injections of ATP or PPADS were centered about 400 μm caudal to the *calamus scriptorius* (Fig. 17B and 18B). A single injection of ATP or PPADS was administered in or near the midline. Based on the distribution of the fluorescent microbeads (Fig. 17B), the injectate spread bilaterally ~ 500 μm from the injection center and ~300 μm from the injection center in the rostrocaudal direction.

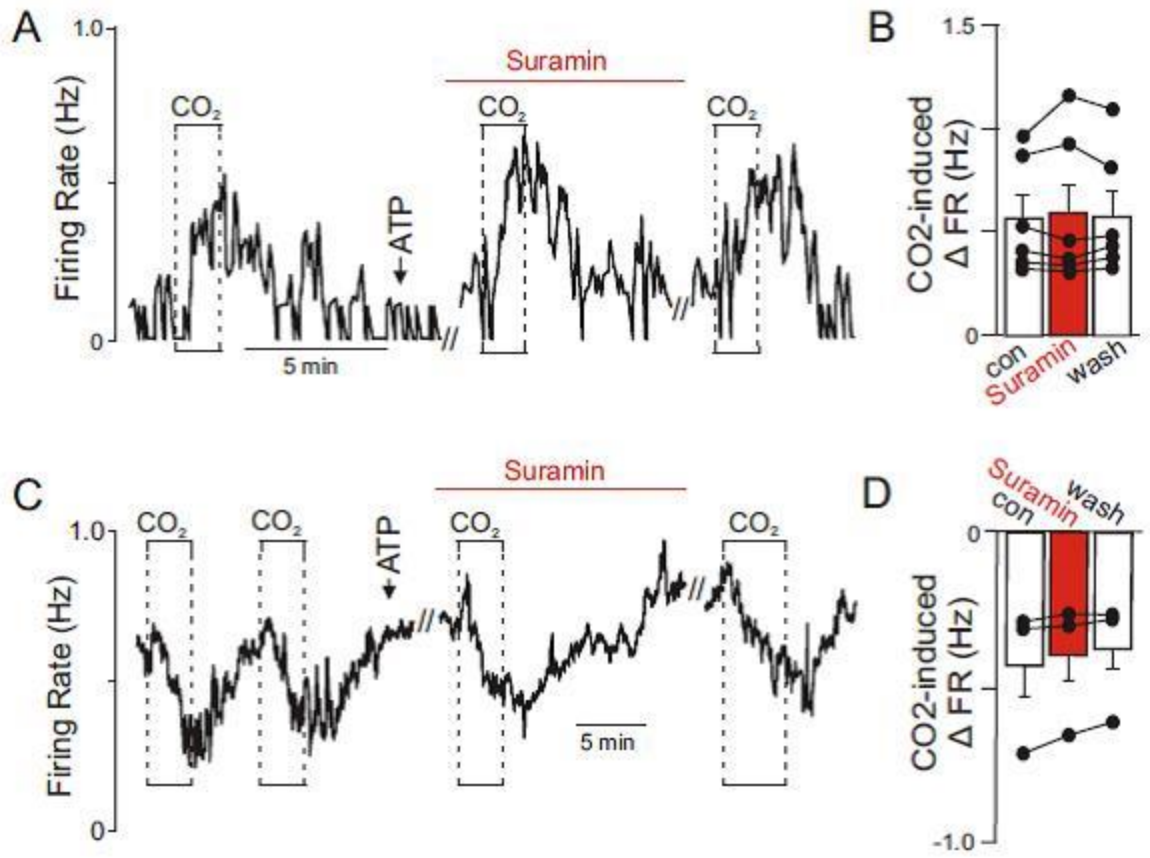
Purinergic signaling in the medullary raphe does not modulate activity of CO_2-sensitive neurons *in vitro* or contribute to the chemoreflex *in vivo*.

We recorded from CO_2/H^+-sensitive medullary raphe neurons in slices to characterize effects of purinergic signaling on baseline activity and CO_2-resonsivness. For these experiments we targeted the raphe pallidus at approximately the same rostrocaudal level as the RTN (-11.6 mm bregma). Cells in this region were considered CO_2/H^+-sensitive (activated or inhibited) if they respond to 15% CO_2 with ≥ 20% change in firing rate (this corresponds with a chemosensitivity index of ≥ 120% or ≤ 80%). This criteria is similar to previous definitions of raphe chemosensitivity (Richerson, 1995; Wang & Richerson, 1999). It should be noted that cells were presumed to be serotonergic based on i) location, ii) CO_2/H^+-sensitivity, and iii) expression of a characteristic serotonin-activated inward potassium conductance (Bayliss *et al.*, 1997) measured in voltage clamp at the end of each experiment, data not shown). The majority of cells in this region did not respond to 15% CO_2, as expected for raphe neurons in slices isolated from animals ≤ 12 days of age (Wang & Richerson, 1999). Raphe neurons that were stimulated by hypercapnia (N=6) had a spontaneous discharge rate of 0.96 ± 0.26 Hz in 5% CO_2 that increased to 1.53 ± 0.33 Hz in 15% CO_2 (Figs. 19A-B). Raphe neurons that were inhibited by hypercapnia (N=3) had a spontaneous firing rat of 0.70 +/- 0.13 in 5% CO_2 that decreased to 0.28 ± 0.06 in 15% CO_2 (Figs. 19C-D).

To determine if purinergic signaling contributes to activity of these cells, we next measured CO_2/H^+-responsiveness of raphe pallidus neurons when P2 receptors are blocked with suramin. Bath application of suramin (100 μM) had no measurable effect on baseline activity or CO_2/H^+-sensitivity of

Figure 19

Purinergic signaling does not modulate activity or CO2/H+-sensitivity of raphe pallidus neurons *in vitro*. A, trace of firing rate from a CO2/H+-activated RPa neuron shows that exposure to 15% CO2 increased firing rate ~0.5 Hz under control conditions and when P2 receptors were blocked with suramin (100 μM). In addition, focal application of ATP (1 mM) had no effect on neuronal activity (arrow). **B,** summary data (N=6) shows that suramin had no effect on the CO_2/H^+-activated RPa neurons. **C**, firing rate trace from a CO2/H+-inhibited RPa neuron shows that exposure to 15% CO2 decreased firing rate by ~ 0.3 Hz under control conditions and during P2 receptor blockade with suramin. Focal application of ATP (1 mM) had no effect on neuronal activity (arrow). **D,** summary data (N=3) shows that suramin had no effect on the CO_2/H^+-inhibited RPa neurons. // designates 10 min time breaks.

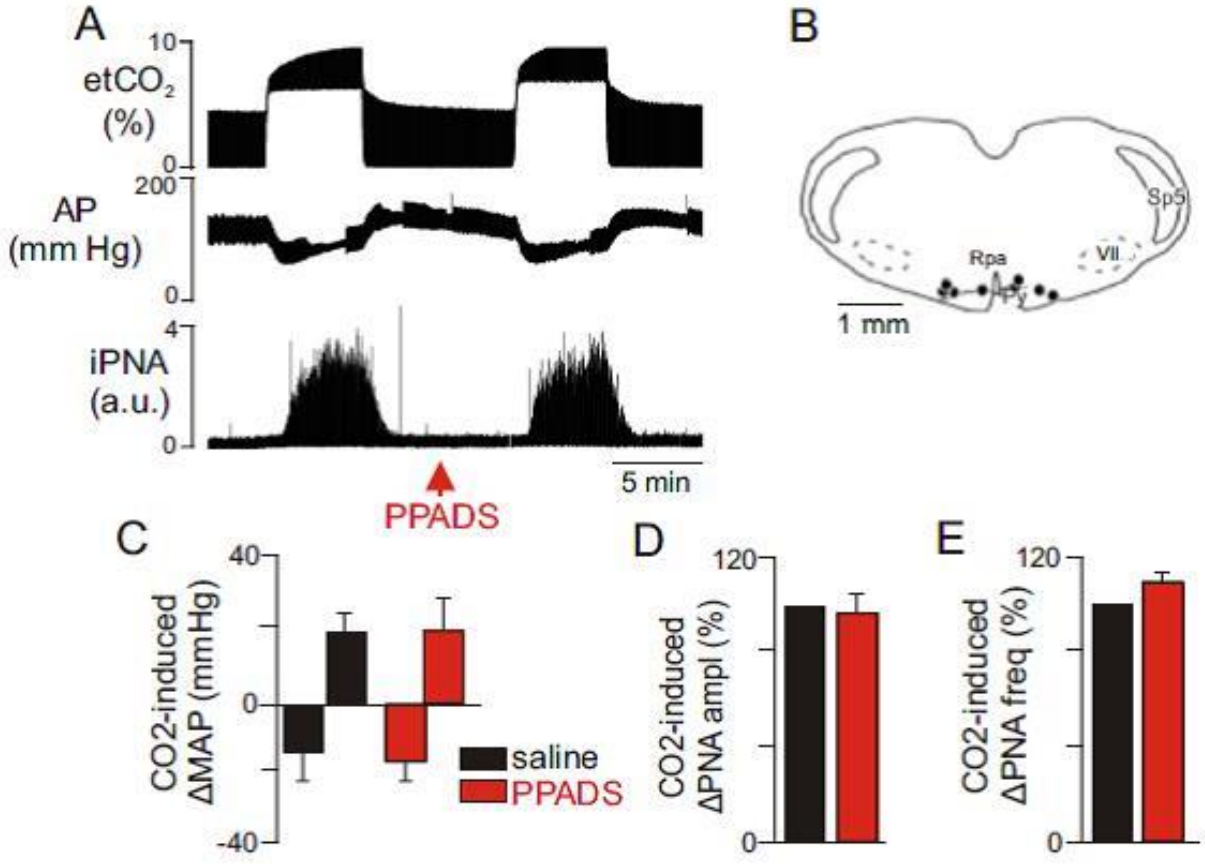

A
1.0
0
Firing Rate (Hz)
Suramin
CO2
CO2
CO2
ATP
5 min
B
1.5
0
CO2-induced
Δ FR (Hz)
con
Suramin
wash
C
1.0
0
Firing Rate (Hz)
Suramin
CO2
CO2
ATP
CO2
CO2
5 min
D
Suramin
con
wash
0
-1.0
CO2-induced
Δ FR (Hz)

these cells. For example, in the presence of suramin CO_2/H^+-activated cells respond to hypercapnia with an increase in firing rate from 1.12 ± 0.36 Hz to 1.74 ± 0.15 Hz (Figs. 19A-B). This response corresponds with an activity increase of 0.60 ± 0.15 Hz which is not significantly different from CO_2/H^+-induced increase under control conditions (0.57 ± 0.11 Hz). Similarly, in the presence of suramin CO_2/H^+-inhibited neurons respond to 15% CO_2 with a decrease in firing rate from 0.76 ± 0.12 Hz to 0.39 ± 0.12 Hz (Figs. 19C-D). This corresponds with a 0.39 ± 0.12 Hz decrease in activity which is similar to CO_2/H^+-mediated inhibition under control conditions (Figs. 19C-D). Furthermore, in a separate series of experiments we found that 11 (7 CO_2/H^+-activated and 6 CO_2/H^+-inhibited) of 13 CO_2/H^+-sensitive raphe pallidus neurons showed no measurable response to focal application of ATP (1 mM in HEPES buffered saline, pH 7.3) (Figs. 19A-C). Two raphe neurons (one CO_2-activated and one CO_2-inhibited) responded to ATP with an increase in firing rate (data not shown). Together, these results indicate that purinergic signaling does not contribute to CO_2/H^+-sensing by raphe pallidus neurons *in vitro*.

To determine if purinergic signaling in the medullary raphe contributes to the whole animal chemoreflex, we tested effects of medullary raphe injections of ATP and PPADS on baseline breathing and the ventilatory response to CO_2 in urethane-anesthetized rats. For these experiments we targeted the raphe pallidus (RPa) (N = 6) and the paraparamidal region (Ppy) (N = 5) because serotonergic cells in region reportedly function as chemoreceptors (Richerson 2004) and they are located within diffusion distance (estimated to be ~400 μm from the ventral surface (Spyer, Gourine 2009) from sites of CO_2/H^+-evoked ATP release on the ventral surface. Injections of PPADS were placed bilaterally in the medullary raphe in these rats (Fig. 20B). The injection center was 200-230 μm below the facial motor nucleus, 200 μm rostral to the caudal end of this nucleus and 1 mm lateral to the midline as previously demonstrated (Mulkey, Stornetta et al. 2004, Takakura, Moreira 2011). Consistent with our *in vitro* data, we found that bilateral injections of PPADS (3 mM - 50 nl) into either the RPa or Ppy had no effect on baseline PNA or the ventilatory response to CO_2. For example, bilateral injections of PPADS (3 mM - 50 nl; N = 8/group) into these did not change resting PNA (101 ± 3% of control, p = 0.246) or MAP (119 ± 3 mmHg

Figure 20

P2 receptor antagonist injections into the medullary raphe had no effect on hypercapnia-induced changes in arterial pressure and PNA in vago-sino-aortic denervated rats. A, recordings from one rat show effects of injection of PPADS into the RPa/Ppy on changes in phrenic nerve activity (PNA) elicited by an increase of end-expiratory CO_2 from 5 to 10%. Responses were recorded 10 min after injection of saline (50 nl) or PPADS (3 mM, 50 nl) into the RPa/Ppy. **B,** computer-assisted plot shows the center of the injection sites (coronal projection on plane Bregma -11.6 mm; (Paxinos, Watson 1982). **C-D**, summary data (N=8) shows that PPADS injection into the RPa/Ppy had no effect on CO2-induced changes in mean arterial pressure (ΔMAP) (C), PND amplitude (ΔPND ampl) (D) or PND frequency (ΔPND freq) (E). Abbreviations: py, pyramid; RPa, raphe pallidus; RPpy, raphe parapyramidal ; Sp5, spinal trigeminal tract; VII, facial motor nucleus. Black dots represent the injections sites in the RPa/Ppy.


A
10
etCO2
(%)
0
200
AP
(mm Hg)
0
4
iPNA
(a.u.)
0
5 min
PPADS
B
Sp5
Rpa
VII
1 mm
C
40
0
-40
CO2-induced
ΔMAP (mmHg)
saline
PPADS
D
120
0
CO2-induced
ΔPNA ampl (%)
E
120
0
CO2-induced
ΔPNA freq (%)

compared to saline 117 ± 4 mmHg, p = 0.234) (Fig. 20A). In addition, bilateral injections of PPADS into these regions did not affect the hypercapnia-induced increase in PNA amplitude (106 ± 11% vs. saline: 105 ± 14%, p = 0.237), PNA frequency (102 ± 8% vs. saline: 101 ± 5%, p = 0.264), or pressor response (22 ± 4 mmHg vs. saline: 22 ± 6 mmHg, p = 0.179) (Fig. 20A-E). Also consistent with our slice data, we found that injections of ATP (10 mM - 50 nl) into the medullary raphe had no effect on PNA ampl, PNA freq and blood pressure (Figs. 21A-E).

Application of ATP into the RTN increases cardiorespiratory activity

There is strong evidence that purinergic signaling contributes to RTN chemoreception (Gourine, Llaudet et al. 2005, Gourine, Kasymov et al. 2010, Huckstepp, id Bihi et al. 2010, Wenker, Kreneisz et al. 2010, Wenker, Sobrinho et al. 2012). We confirm this possibility by testing effects of unilateral injections of ATP into the RTN on cardiorespiratory activity. These experiments also serve as a positive control for similar experiments in the commNTS and medullary raphe (described above). Consistent with previous evidence (Gourine, Llaudet et al. 2005, Mulkey, Mistry et al. 2006), we found that unilateral injection of ATP into the RTN increased MAP (14 ± 3, vs. saline: 2 ± 3 mmHg, p = 0.034), PNA amplitude by 26 ± 5% and PNA frequency by 21 ± 4% (Fig. 22A-E). These results confirm that purinergic signaling at the level of the RTN regulates cardiorespiratory activity.

The RTN contains a higher density of protoplasmic astrocytes compared to the cNTS or RPa.

Previous evidence suggests that CO_2-evoked ATP release from astrocytes contributes to RTN chemoreception (Gourine, Kasymov et al. 2010, Huckstepp, id Bihi et al. 2010, Wenker, Kreneisz et al. 2010, Wenker, Sobrinho et al. 2012). In the present study we show that purinergic signaling does not contribute to chemoreception in the commNTS or medullary raphe. We postulate that the differential role of purinergic signaling in these regions results from a higher density of astrocytes in the RTN compared to the commNTS or medullary raphe. To test this possibility, we determine the relative density of astrocytes by counting the number of

Figure 21

Injection of ATP into the medullary raphe had no effect on breathing or blood pressure. A, recordings of AP and PNA show that application of ATP (10 mM, 50 nl) into the medullary raphe (RPa/Ppy) did not affect blood pressure or respiratory motor output. **B,** histology section showing the distribution of fluorescent microbeads within the RPa. **C-E**, summary data (N=11) show that RPa/Ppy injections of ATP had no effect on mean arterial pressure (ΔMAP) (C), PND amplitude (ΔPND ampl) (D) or PND frequency (ΔPND freq) (E). Scale bar in B is 50 μm.

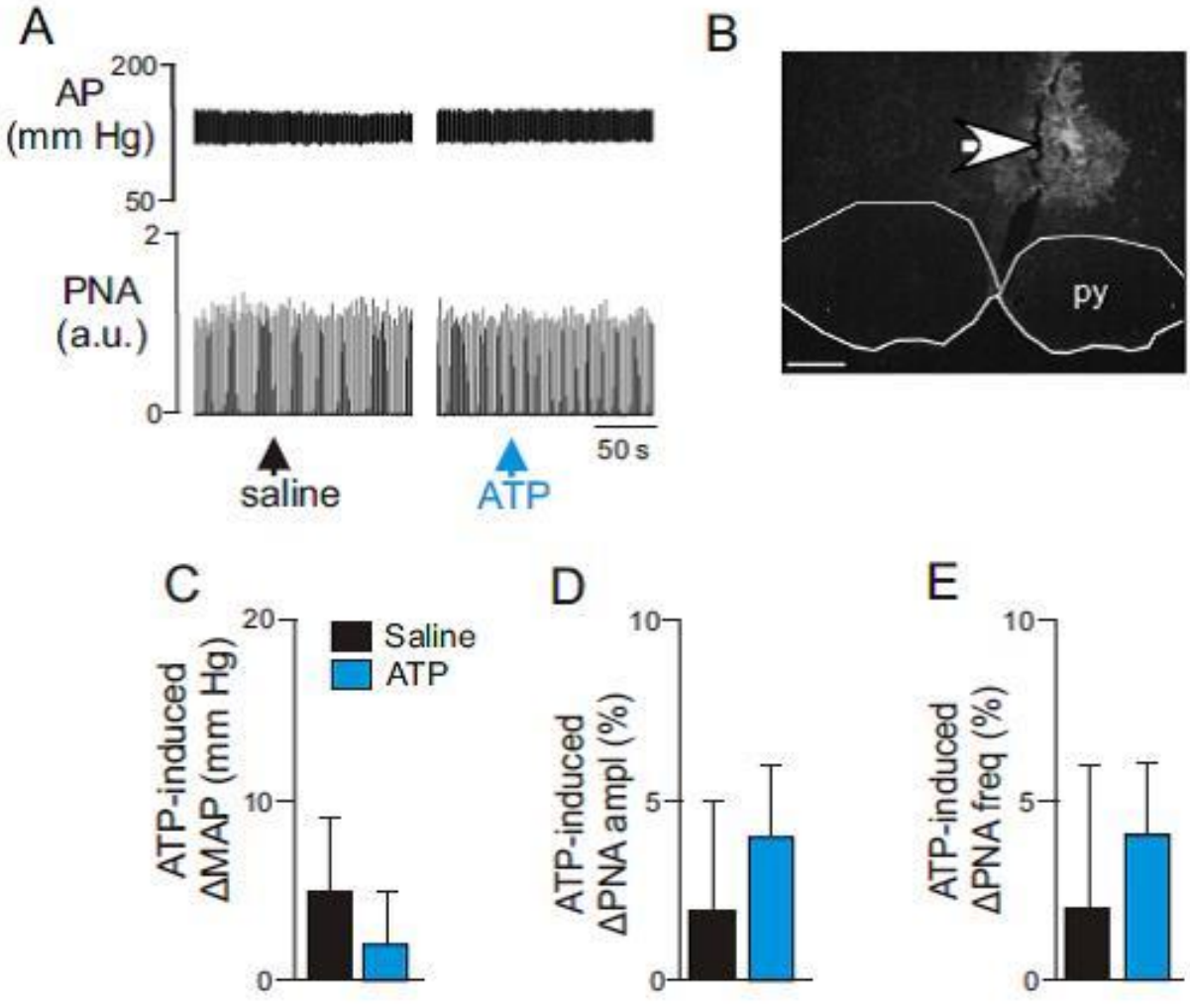
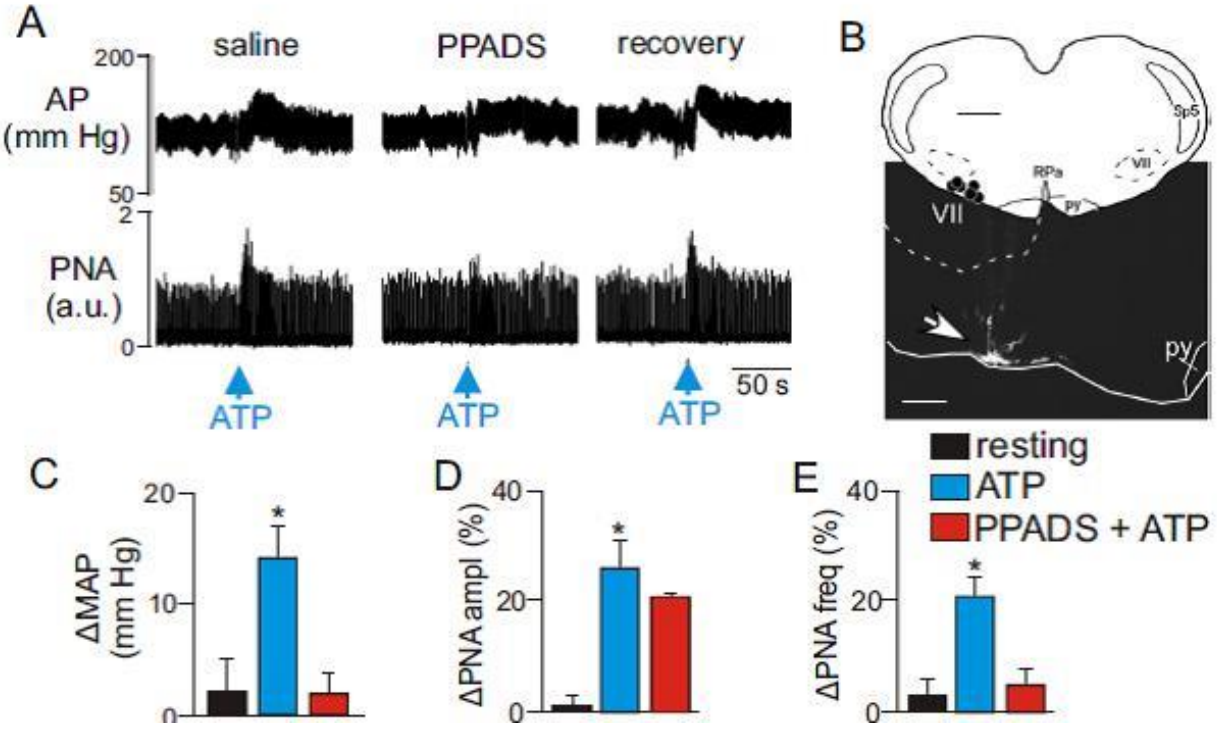

A
AP
(mm Hg)
200
50
PNA
(a.u.)
2
0
50 s
saline
ATP
B
py
C
ATP-induced
ΔMAP (mm Hg)
20
10
0
Saline
ATP
D
ATP-induced
ΔPNA ampl (%)
10
5
0
E
ATP-induced
ΔPNA freq (%)
10
5
0

Figure 22

ATP injections into the RTN increase cardiorespiratory activity. A, recordings of arterial pressure (AP) and phrenic nerve activity (PNA) show that under control conditions unilateral injection of ATP (10 mM, 50 nl) into the RTN increased breathing and blood pressure. Injection of PPADS (3 mM, 50 nl) into the RTN blocked cardiorespiratory responses to subsequent applications of ATP. **B,** computer-assisted plot and representative histological section show center of injection sites (coronal projection on plane Bregma -11.6 mm; (Paxinos, Watson 1982)). **C-D**, summary data (N=6) show changes in mean arterial pressure (ΔMAP) (B), PND amplitude (ΔPND ampl) (C) and PND frequency (ΔPND freq) (D) elicited by injection of saline or ATP into the RTN. Abbreviations: py, pyramid; RPa, raphe pallidus; Sp5, spinal trigeminal tract; VII, facial motor nucleus. Black dots represent the injections sites in the RPa/Ppy. Top and bottom scale bars in B are 1 mm and 50 µm, respectively.


A
saline
PPADS
recovery
200
AP
(mm Hg)
50
2
PNA
(a.u.)
0
ATP
ATP
ATP
50 s
B
Sp5
RPa
VII
py
VII
py
C
ΔMAP
(mm Hg)
20
10
0
*
D
ΔPNA ampl (%)
40
20
0
*
E
ΔPNA freq (%)
40
20
0
*
resting
ATP
PPADS + ATP

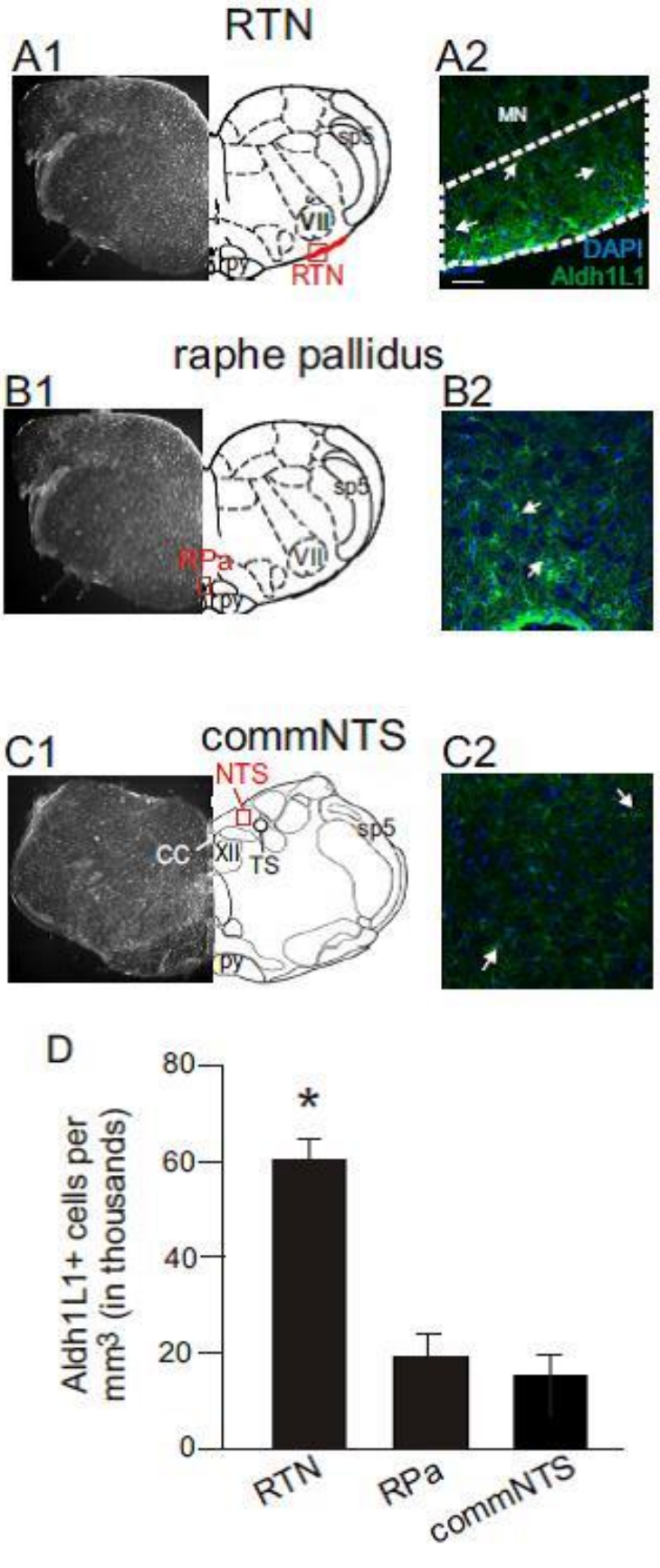
Aldh1L1-positive cells in a 1 mm^2 section within the regions of interest (see methods for details). Astrocytes were counted in confocal Z-stack images by identifying DAPI-labeled nuclei with greater than 50% surrounded by Aldh1L1 staining. Images of the RTN were taken in a region located between the pyramids and trigeminal and directly ventral to the facial motor nucleus at a depth ~100 μm from the ventral surface (Fig. 23A). Images of the raphe pallidus were taken midline between the pyramids and near the ventral surface (Fig. 23B). Images of the commNTS were taken medial to the solitary tract in slices that were ~200 μm caudal to area postrema (-14.04 mm Bregma) (Fig. 23C). We found that the RTN contained approximately 3 times as many astrocytes as the commNTS or raphe pallidus (Fig. 23D).

DISCUSSION

There is compelling evidence that CO_2/H^+-evoked ATP release from ventral surface astrocytes contributes to respiratory drive by activating chemosensitive RTN neurons (Gourine, Llaudet et al. 2005, Wenker, Sobrinho et al. 2012, Huckstepp, id Bihi et al. 2010). Here, we confirm that activation of P2-receptors in the RTN increases cardiorespiratory activity, and we test at the cellular and systems level whether purinergic signaling also modulates activity or CO_2/H^+-sensitivity of neurons in two other brainstem regions thought to contribute to central chemoreception (i.e., the commNTS and medullary raphe). As expected, application of ATP into the RTN elicited a strong increase in breathing and a mild increase in blood pressure by a P2-receptor dependent mechanism, thus further supporting the possibility that purinergic signaling in the ventrolateral medulla can modulate activity of respiratory chemoreceptors (Gourine, Llaudet et al. 2005, Wenker, Sobrinho et al. 2012, Huckstepp, id Bihi et al. 2010) and blood pressure regulating C1 cells (Wenker, Sobrinho et al. 2013). Similar to previous evidence in the RTN, CO_2/H^+-sensitive commNTS neurons also respond to focal ATP application with a robust increase in firing rate, and *in vivo* ATP injection into the commNTS increased breathing. Conversely, CO_2/H^+-sensitive medullary raphe neurons did not respond to ATP nor did injection of ATP into this region elicit a cardiorespiratory response. Contrary to evidence in the RTN, inhibition of P2-receptors in the

Figure 23

The RTN has a higher density of astrocytes than the commNTS or RPa. A-C, diagram (right) and photomicrograph (left) of a transverse medullary slices containing the RTN (A1), RPa (B1) and the commNTS (C1). Areas labeled in red represent the regions of interest. Red boxes identify regions of interest where confocal images were taken. **A2-C2,** projection images of confocal z-stacks of the RTN (A2), RPa (B2) and NTS (C2), in which the blue channel represents DAPI staining and the green channel Aldh1L1 immunoreactivity. To be counted, a cell had to have a clearly defined nuclei with bright DAPI staining as well as bright Aldh1L1 staining around at least half of the nucleus (arrows in each image). **D**, summary data (N=4 animals) show the relative astrocyte density in the RTN, RPa and commNTS. Note that the RTN had a significantly higher astrocyte density compared to the commNTS or RPa (one-way ANOVA on ranks). These results support the possibility that astrocytes are an important component of RTN chemoreception.


RTN
A1
A2
MN
sp5
VII
py
RTN
DAPI
Aldh1L1
raphe pallidus
B1
B2
sp5
RPa
VII
py
commNTS
C1
C2
NTS
sp5
cc
XII
TS
py
D
Aldh1L1+ cells per mm3 (in thousands)
80
60
40
20
0
*
RTN
RPa
commNTS

commNTS and medullary raphe had no effect on basal activity and CO_2/H^+-sensitivity *in vitro,* or baseline cardiorespiratory activity and CO_2-responsivness *in vivo*. Furthermore, we found that the RTN contains a higher density of astrocytes compared to the commNTS and raphe pallidus, suggesting that the unique role of purinergic signaling in the RTN is a consequence of astrocyte density. Therefore, previous and present results suggest that purinergic signaling is a unique feature of RTN chemoreception.

The RTN is an important sight of chemoreception and intense effort has been made to identify a unique CO_2/H^+-sensing mechanism in this region. Evidence indicates that chemosensitive RTN neurons sense and respond to changes in tissue CO_2/H^+ directly (Lazarenko, Milner et al. 2009, Onimaru, Ikeda et al. 2012, Wang, Shi et al. 2013), possibly by inhibition of a K^+ conductance (Mulkey, Stornetta et al. 2004, Mulkey, Talley et al. 2007), and indirectly by CO_2/H^+-evoked ATP release most likely from astrocytes (Gourine, Llaudet et al. 2005, Wenker, Sobrinho et al. 2012, Huckstepp, id Bihi et al. 2010). The identity of the neuronal pH-sensor remains unknown and thus precludes further analysis; however, evidence does suggest that a subset of RTN astrocytes are uniquely specialized to release ATP in a CO_2/H^+-dependent manner. For example, CO_2/H^+-evoked ATP release has been shown to occur at discrete regions on the ventral surface near the RTN but not in deeper brainstem regions or near the dorsal surface (Gourine, Llaudet et al. 2005, Huckstepp, id Bihi et al. 2010). In addition, evidence suggests that a subset of ventral surface astrocytes but not cortical astrocytes respond to H^+ with an increase in intracellular Ca^{2+} and vesicular release of ATP (Kasymov, Larina et al. 2013). The results presented here build on the possibility that purinergic signaling is a unique component of RTN chemoreception by showing that P2-recepotr blockade in two putative chemoreceptor regions did not affect CO_2-responsivness *in vitro* or *in vivo*. The differential role of purinergic signaling CO_2/H^+-sensing by cells in these regions may result from a higher density of astrocytes in the RTN compared to the commNTS and medullary raphe; however it is also possible that RTN astrocytes are unique and functionally specialized to release ATP during hypercapnia as previously suggested (Kasymov, Larina et al. 2013).

The caudal portion of the NTS is thought to contribute to central chemoreception because a subset of neurons in this region respond to CO_2 with increased firing rate (Conrad, Nichols et al. 2009, Dean, Bayliss et al. 1990) and focal acidification of this region has been shown to stimulate breathing in anesthetized and unanesthetized animals (Li, Nattie 2002, Coates, Li et al. 1993). Although the cellular and molecular identity of CO_2/H^+-sensors in the commNTS are unknown, it is well established that cells in this region, including Phox2b immunoreactive neurons (Stornetta, Moreira et al. 2006), relay peripheral chemosensory inputs to other components of the respiratory circuit; thus cells in this area are well positioned to regulate cardiorespiratory activity in response to hypercapnia.

Previous evidence suggests that purinergic signaling in the commNTS influences cardiorespiratory activity. For example, several types of P2-receptors are expressed in the commNTS (Yao, Barden et al. 2000) and injection of ATP or related analogs into the commNTS of awake rats mimicked cardiorespiratory responses (i.e., bradycardia, hypertension and tachypneia) to peripheral chemoreceptor activation (Antunes, Braga et al. 2005, de Paula, Antunes et al. 2004, Paton, De Paula et al. 2002). Purinergic signaling also contributes to pulmonary stretch receptor-mediated activation of second-order relay neurons in the NTS to control inspiration (Gourine, Dale et al. 2008). Consistent with the possibility that purinergic signaling in the commNTS contributes to cardiorespiratory control, we show that ATP injections into this region increased cardiorespiratory activity. We also show that CO_2/H^+-sensitive commNTS neurons are strongly activated by focal ATP application. It should be noted that the neurochemical phenotype of CO_2/H^+-sensitive commNTS neurons remains unknown, since these cells were immune-negative for Phox2b and ChAT. Nevertheless, blocking P2-recepotrs in this region did not affect the firing rate response to CO_2/H^+ *in vitro*, or the ventilatory response to CO_2 *in vivo*. These results indicate that purinergic signaling can increase activity of CO_2/H^+-sensitive commNTS neurons and stimulate cardiorespiratory activity, but this mechanism does not contribute to CO_2-responsivness. It remains possible that astrocytes in this region contribute to CO_2/H^+-responsiveness of commNTS neurons, albeit by an ATP-independent mechanism. For example, recent evidence showed that

acidification decreased glutamate uptake by NTS astrocytes and facilitated gutamatergic slow excitatory potentials that could potentially increase integrated output of the NTS to the rest of the respiratory network (Huda, McCrimmon et al. 2013).

Serotonergic medullary raphe neurons can strongly influence breathing (Hodges, Richerson 2010) and are thought to function as respiratory chemoreceptors in part because neurons in this area have been shown to be acid sensitive *in vitro* (Wang, Tiwari et al. 2001, Wang, Pizzonia et al. 1998, Severson, Wang et al. 2003, Richerson 1995), focal acidification of this region increased breathing in unanesthetized awake (Hodges, Martino et al. 2004) or sleeping (Nattie, Li 2001) animals, and genetic deletion (Hodges *et al.*, 2008) or selective inhibition (Ray, Corcoran et al. 2011) of serotonergic neurons decreased the ventilatory response to CO_2. As previously described (Richerson 1995, Wang, Pizzonia et al. 1998) , we found a subset of medullary raphe neurons in slices from neonatal rat pups that responded to 15% CO_2 with a modest increase or decrease if firing rate.

There is some evidence that CO_2/H^+-sensitivity of raphe neurons is partially dependent on purinergic signaling. For example, a study in anesthetized rats showed that microinjection of ATP into the raphe magnus and pallidus decreased or increased respiratory activity, respectively (Cao, Song 2007). In addition, injection of PPADS into the medullary raphe blunted the ventilatory response to CO_2 of conscious rats (da Silva, Moraes et al. 2012). Considering that P2-receptors are ubiquitously expressed in the central nervous system including at the level of the medullary raphe, we were surprised to find that CO_2/H^+-sensitive neurons (i.e., activated or inhibited) in the raphe pallidus did not respond to focal application of ATP *in vitro*, and injection of ATP into the raphe pallidus or the parapiramidal region had no effect on baseline breathing *in vivo*. In addition, blocking P2-receptors in these medullary raphe regions did not perturb CO_2-sensitivity *in vitro* or *in vivo*. These results suggest that purinergic signaling does not influence activity or CO_2-sensitivity at these raphe nuclei under our experimental conditions.

In summary, purinergic signaling contributes to the mechanism by which RTN neurons sense and respond to changes in CO_2/H^+; however, this signaling mechanism does not contribute to CO_2/H^+-sensing in the commNTS or medullary raphe. These results are the first evidence for a unique CO_2/H^+-sensing mechanism in the RTN.

Chapter 7 Summary of Major Findings

Studies from the late 1990's and early 2000's indicated that purinergic signaling in the ventrolateral medulla is required for proper chemoreception (Thomas, Ralevic et al. 1999, Thomas, Spyer 2000, Gourine, Llaudet et al. 2005). This prompted us to ask the question, what role purinergic signaling plays in chemoreception at the cellular level? The results of this thesis support a model in which CO_2-evoked ATP is released from astrocytes via connexin hemichannels to activate RTN neurons and increase respiratory activity. This provides valuable insight into the fundamental mechanisms of central chemoreception. This thesis reaches four main conclusions. (1) One mechanism for sensing CO_2/H^+ by astrocytes in by inhibition of Kir4.1-Kir5.1-like channels and the sodium bicarbonate cotrasporter. (2) Functional purinergic signaling is required for proper chemoreception, both *in vivo* and *in vitro*, and CO_2, not pH per se, is the stimulus. (3) The method for ATP release due to CO_2/H^+ is opening of connexin hemichannels. (4) The contribution of purinergic signaling to neuronal CO_2/H^+ sensitivity is restricted to the RTN/medullary ventral surface. Below, I will discuss these results with respect to the current literature and propose future experiments to more decisively answer the above questions.

Conclusions

(1) A number of previous reports demonstrate functional Kir4.1 channels in astrocytes of the cortex (Higashi, Fujita et al. 2001, Olsen, Higashimori et al. 2006, Tang, Taniguchi et al. 2009). These channels are active at negative potentials and contribute to the very negative membrane potential observed in all astrocytes. In addition Kir4.1 channels are thought to mediate potassium buffering during high neuronal activity (Djukic, Casper et al. 2007, Olsen, Sontheimer 2008). Thus, our observation that Kir4.1-Kir5.1 channels are expressed in astrocytes is by no means unexpected. However, it is unusual to find such a large pH-sensitive conductance in astrocytes. In the hippocampus, more extreme pH solution (compared to the present study) resulted in only mild inhibition of resting potassium current (Zhou, Xu et al. 2009). On the other hand, RTN astrocytes decreased membrane conductance by 50% on average. This translates

to approximately a 10 mV decrease in membrane potential, which is enough to activate voltage gated channels, including certain connexins (Saez, Retamal et al. 2005). The physiological consequences of this astrocytic sensing mechanism are not known. Difficulties with pharmacology prevented us for determining the role of Kir4.1 in chemoreception *in vivo*. Others have found that congenital KO of Kir5.1 (necessary for physiological pH sensing by Kir4.1) does not affect the central chemoreflex (Trapp, Tucker et al. 2011). However, the animals suffer from metabolic acidosis, which is intimately entwined with sensation of blood gases, and thus could offset any slight change to chemosensitivity. To more completely answer this question it is likely that conditional genetic ablation of Kir4.1 or Kir5.1 is needed (see future directions).

(2) The most logical mechanism for ATP released at the medullary ventral surface to affect respiratory output, is for the ATP to activate local respiratory neurons which then relay this information to the proper respiratory centers. The alternative, diffusion of ATP to the respiratory CPG, seems unlikely, as the distance is great and ATP would likely be broken down by ectonucleotidases in the process. In addition, ATP applied to the prebotzinger complex results in a biphasic respiratory response (Lorier, Huxtable et al. 2007), very dissimilar to the response mediated by the central chemoreflex. It was already known that ATP could increase action potential activity in RTN, but it was also shown that blockade of P2 blockers had no effect on the pH-sensing of the same neurons (Mulkey, Mistry et al. 2006). In the present study we found different results when using bicarbonate buffered external solution and decreased pH by elevating CO_2 concentration. RTN neuronal CO_2/H^+ sensitivity was reduced by ~25% when P2 receptors were blocked. This effect was independent of external calcium, synaptic input, temperature and stimulus strength. Independent evidence exists that demontstrates changes in CO_2, but not necessarily pH, are necessary for ATP release (Huckstepp, Eason et al. 2010, Huckstepp, id Bihi et al. 2010), although they did not assay neuronal function. However, other studies have demonstrated ATP release by altering pH independent of CO_2 (Gourine, Kasymov et al. 2010). In addition, these studies found that in the absence of functional P2 receptors neurons near the ventral surface lacked CO_2/H^+ chemosenstivity entirely. The

reason for the discrepancies between this and the present study is unclear, but a number of experimental differences are likely the culprit. In Gourine et al., the authors use organotypic slices, as opposed to acute slices, for whole-cell recordings, as opposed to loose-patch recordings, and use a synthetic Phox2a/b promoter driving a reporter to identify RTN neurons, as opposed to identification based on CO_2/H^+-sensitivity used in this study. The synthetic promoter is not specific for central chemoreceptor RTN neurons, with expression being seen in other nearby neurons, including the C1 (Abbott, Stornetta et al. 2009), which could explain the lackluster neuronal chemosensitivity and difference in P2 receptor function. One thing in agreement between all groups (Huckstepp, id Bihi et al. 2010, Gourine, Kasymov et al. 2010, Wenker, Sobrinho et al. 2012), is that P2 receptor antagonism in the RTN results in decreased *in vivo* chemosensitivity by ~25-30%.

(3) There is consensus that astrocytes in the RTN release ATP, but the mechanism of release is debated (Gourine, Kasymov et al. 2010, Huckstepp, id Bihi et al. 2010). The two competing hypotheses are (a) calcium-mediated vesicular release and (b) direct CO_2 gating of connexin channels (specifically Cx26) allowing flux of ATP. In this thesis we assayed for mechanisms of ATP release by using the RTN neuronal response to CO_2/H^+ as a readout for ATP release. We found that using a low calcium, high magnesium, solution to block vesicular release had no effect on CO_2/H^+ sensitivity of RTN neurons. However, pharmacological blockade of connexin hemichannels has similar effects on chemosensitivity as did P2 receptor antagonists. In addition, simultaneous antagonism of connexins and P2 receptors had the same effect as either one alone, indicating they are both involved in the same signaling pathway. Once again, our data best supports that of Huckstepp et al. (2010), but does not sync well with work by Gourine et al (2010). Possible explanation for these discrepancies are the same as described above in conclusion (2), and likely the different findings related to purinergic signaling's mechanism of release, effect on neuronal chemosensitivity and molecule sensed (i.e. H^+ versus CO_2) are all related.

(4) Initial experiments found ATP released *in vivo* due to hypercapnia was restricted regionally to ventral surface of the medulla (Gourine, Llaudet et al. 2005). In horizontal brain slices, Huckstepp et al. (2010) also observed ATP release was restricted somewhat in the lateral direction, but also noted that slices dorsal to the first slice did not exhibit any ATP release during hypercapnia. This prompted us to wonder whether purinergic signaling is a mechanism of CO_2/H^+ sensing that is specific to RTN neurons. The search for specific sensing mechanisms is not new (Guyenet, Stornetta et al. 2008). In line with previous studies, we found that purinergic signaling is restricted to RTN neurons. Neither medullary raphe nor cNTS chemosensitive neurons rely on P2 receptors for their CO_2/H^+ sensing abilities. For cNTS neurons, this is in spite of the fact that they do express functional P2 receptors. Interestingly, we did not detect functional P2 receptor expression in Raphe neurons. These data provide evidence that purinergic signaling involvement in central chemoreception is restricted to the RTN.

Future studies

The bulk of the experiments in this thesis rely on pharmacological manipulations. As I was once told, "A drug seems to lose its specificity with age." This is not advice against keeping a drug in storage for an extended period of time, but it points out that the specificity of a pharmacological agent tends to lessen as we learn more about the agent. Lack of specificity aside, using pharmacology to study astrocytes is problematic. Astrocytes are cells just like neurons. They express many of the same channels and receptors, use calcium as an intracellular signaling mechanism and can release transmitters to affect other glial cells and neurons (Kimelberg 2010). Any attempt to use pharmacology to antagonize a mechanism in astrocytes will simultaneous antagonize the same mechanism in neurons, making it impossible to dissect which cells are responsible for an action. In the present study we do our best to work around this but it is inevitable. Future studies should utilize conditional genetic approaches (e.g. GFAP-cre expressing mice, or targeted virus mediated infection) to affect astrocytes selectively. Logical next steps would include astrocyte specific knock out of genes that encode for Kir4.1, Kir5.1, Cx26, IP3 receptors and astrocyte SNARE proteins. Some of these genetic tools have been very useful in the Cortex (Djukic,

Casper et al. 2007, Petravicz, Fiacco et al. 2008, Pascual, Casper et al. 2005). The same can be said for neurons. This thesis has provided evidence that RTN neurons express functional P2Y receptors. However, the molecular identity (i.e. receptor subtype) has been difficult to determine (results not shown). This may be best determined using similar conditional genetic manipulations in neurons. Although no system is perfect, tools for relatively specific targeting to RTN neurons exist that could allow for targeted antagonism of specific P2Y receptor subtypes. Let us also not forget that ~75% of RTN neuronal chemosensitivity appears to be intrinsic, with evidence of a background pH-sensitive potassium channel. Identification of this channel(s) and its sensing mechanism are necessary for complete understanding of RTN chemoreception. Once these precise mechanisms can be targeted efficiently we may be able know the exact mechanism of central chemoreception in a behaving animal.

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Chapter 9 Curriculum Vitae

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EDUCATION

2008-2013 **University of Connecticut, Department of Physiology and Neurobiology, Storrs, CT**
Ph.D. Candidate (Expected graduation December 2013)
- Thesis: Purinergic regulation of central and peripheral chemoreception in the retrotrapezoid nucleus.
- GPA: 3.96

2006-2008 **Wright State University, Department of Neuroscience, Cell Biology and Physiology, Dayton, OH**
M.S. (Graduation 2009)
- Thesis: P2Y receptor function is necessary, but not sufficient, for hypoosmotic activation of $I_{Cl,swell}$.
- GPA: 4.0

2001-2005 **Xavier University, Department of Biology, Cincinnati, OH**
B.S. (Graduation 2005)
- GPA: 3.45

AWARDS

2011 **Best graduate student scientific presentation**, Physiology and Neurobiology, *University of Connecticut*.
2008 **Student Presenter**, 2008 Ohio Miami Valley Neuroscience Day, *Wright State University*.
2008 **Graduate Student Excellence Award**, *Wright State University*.

PROFESSIONAL SOCIETIES

2012-Present **American Physiological Society**
2008-Present **Society for Neuroscience**

SCHOLARSHIPS, FELLOWSHIPS AND GRANTS

2013-Present **Neuroscience Fellowship Awardee**, University of Connecticut
2011-2013 **American Heart Association Predoctoral Fellowship** (11PRE7580037), Founder's Affiliate.
2008-2011 **Outstanding Scholars Fellowship**, School of Graduate Studies, *University of Connecticut*.
2006-2008 **Academic Scholarship**, *Wright State University*.

TEACHING EXPERIENCE

2013	**Certificate in College Instruction** from the University of Connecticut
3 Semesters	**Teaching assistant**, Physiology and Anatomy Laboratory *University of Connecticut, Storrs*
2 Semesters	**Teaching assistant**, Investigations in Neurobiology *University of Connecticut, Storrs*
1 Lecture	**Guest lecturer**, Biology of the Brain *University of Connecticut, Storrs*

REFERENCES

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PEER-REVIEWED PUBLICATIONS

1. **Wenker IC***, Sobrinho CR*, Takakura AC, Mulkey DK, Moreira TS. P2Y1 receptors expressed by C1 neurons determine peripheral chemoreceptor modulation of breathing, sympathetic activity, and blood pressure. *Hypertension*. (2013) [Epub ahead of print].

2. Hawryluk J, Moreira TS, Takakura AC, **Wenker IC**, Tzingounis A, and Mulkey DK. KCNQ channels determine serotonergic modulation of ventral surface chemoreceptors and respiratory drive. *Journal of Neuroscience*. (2012) 32: 16943-52.

3. **Wenker IC**, Sobrinho CR, Takakura AC, Moreira TS, Mulkey DK. Regulation of ventral surface CO_2/H^+-sensitive neurons by purinergic signaling. *Journal of Physiology.* (2012) 590: 2137-50.
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4. **Wenker IC***, Benoit JP*, Chen X, Liu H, Horner RL, Mulkey DK. Nitric oxide activates hypoglossal motoneurons by cGMP -dependent inhibition of TASK channels and cGMP-independent activation of HCN channels. *Journal of Neurophysiology*. (2011) 107: 1489-99.

5. Mulkey DK, **Wenker IC**. Astrocyte chemoreceptors: Mechanisms of H+-sensing by astrocytes in the retrotrapezoid nucleus and their possible contribution to respiratory drive. *Experimental Physiology*. (2010) 96 (4): 400-6.

6. **Wenker IC***, Kréneisz O*, Nishiyama A, Mulkey DK. Astrocytes in the retrotrapezoid nucleus sense H+ by inhibition of a Kir4.1-Kir5.1-like current and may contribute to chemoreception by a purinergic mechanism. *Journal of Neurophysiology*. (2010) 104 (6):3042-52.

7. **Wenker I**. An active role for astrocytes in synaptic plasticity? *Journal of Neurophysiology*. (2010) 104 (3):1216-8.

8. Mulkey DK, **Wenker IC**, Kréneisz O. Current ideas on central chemoreception by neurons and glial cells in the retrotrapezoid nucleus. *Journal of Applied Physiology*. (2010) 108 (5):1433-9.

9. McCarthy MC, Moncrief H, Sands JM, Markert RJ, Hall LC, **Wenker IC**, Anderson HL 3rd, Ekeh AP, Walusimbi MS, Woods RJ, Saxe JM, Tchorz KM. Neurologic outcomes with cerebral oxygen monitoring in traumatic brain injury. *Surgery*. (2009) 146 (4):585-90.

MANUSCRIPTS IN PREPARATION

Sobrinho CR*, **Wenker IC***, Poss EM, Takakura AC, Moreira TS, Mulkey DK. Regional specificity of purinergic signaling and astrocytes in central chemoreception. [Experimental Biology 2013 abstract number 5201]

INTERNATIONAL CONFERENCE ORAL PRESENTATIONS

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Benoit JP, Kreneiz O, **Wenker IC**, Mulkey DK. Nitric oxide (NO) inhibits TASK channels and occludes the effects of serotonin on hypoglossal motoneurons in rat brain slices. Program No 89.22, Abstract Planner Soc Neurosci, 2009.

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Wenker IC, Takakura A, Moreira T, Mulkey DK. P2Y1-receptors are expressed by CO_2/H^+-insensitive neurons in the retrotrapezoid nucleus and contribute to the peripheral drive to breathe. Program No. 94.2 Abstract Planner Experiential Biology 2012.

Wenker IC, Chen X, Liu H, Horner R, Mulkey DK. Nitric oxide activates hypoglossal motoneurons by cGMP-dependent inhibition of task channels and cGMP-independent activation of HCN channels. Program No. 1147.6 Abstract Planner Experiential Biology 2012.

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Wenker IC, Poss EM, Sobrinho CR, Takakura AC, Moreira TS, Mulkey DK. Purinergic signaling contributes to CO2-sensitivity of neurons in the retrotrapezoid nucleus but not nucleus of the solitary tract or medullary raphe Program No. 656.08. Neuroscience Meeting Planner. San Diego, CA: Society for Neuroscience, 2013. Online.

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